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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 8755
GLOTZER *et al.*	Art Unit: 1653
Appl. No. 09/881,736	Examiner: *To Be Assigned*
Filed: June 18, 2001	Atty. Docket: 0652.2260001/EKS/AES
For: **Cyk-4 Polypeptides, DNA Molecules Encoding Them and Their Use in Screening Methods**	

Submission of Certified Copy of 35 U.S.C. § 119(a)-(d) Priority Document In Utility Application

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Sir:

Submitted herewith are certified copies of Applicants' U.S.C. § 119(a)-(d) priority documents, to perfect the claim to priority filed on June 18, 2001.

Country	Priority Document Appl. No.	Filing Date
Europe	00 112 880.0	June 19, 2000
Europe	01 110 554.1	April 30, 2001

Prompt acknowledgment of this submission is respectfully requested.

Respectfully submitted,

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Date: December 21, 2001

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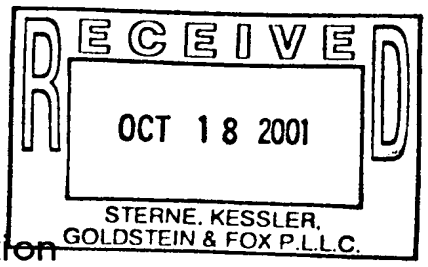

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Patentanmeldung Nr. Patent application No. Demande de brevet n°

00112880.0

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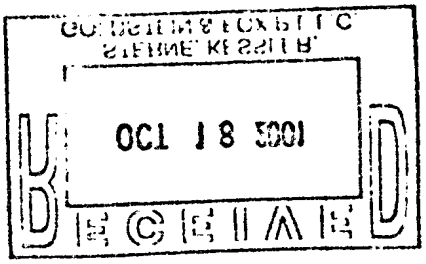
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Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.: 00112880.0
Demande n°:

Anmeldetag:
Date of filing: 19/06/00
Date de dépôt:

Anmelder:
Applicant(s):
Demandeur(s):
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Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
Cyk-4 polypeptides, DNA molecules encoding them and their use in screening methods

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: State: Pays:	Tag: Date: Date:	Aktenzeichen: File no. Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:
C12N15/12, C07K14/47, A01K67/033, C12N15/00

Am Anmeldetag benannte Vertragstaaten:
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE/TR
Etats contractants désignés lors du depôt:

Bemerkungen:
Remarks:
Remarques:

Case 14/057 DI Fa/dc

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Cyk-4 polypeptides, DNA molecules encoding them and their use in screening methods

The present invention relates to a new protein Cyk-4, which is involved in cytokinesis, and to therapies interfering with cell division, in particular tumor therapy.

The process of cytokinesis produces two daughter cells from a single parental cell and permanently segregates the products of the cell division cycle. Cytokinesis is one of the few processes in biology known to require coordination between microtubules and actin filaments. Indeed, in animal cells there are two steps in cytokinesis that rely on interactions between microtubules and the actin-based contractile ring (for review see (Field et al., 1999; Glotzer, 1997). In the first instance, the microtubule-based mitotic spindle specifies the position of the contractile ring. This allows the division plane to be positioned so that the separated chromosomes are partitioned equally into the two daughter cells. Subsequently, after ingression of the cleavage furrow, there is a second step that depends on both microtubules and the contractile ring. Completion of cytokinesis requires the central spindle, which contains bundled, antiparallel microtubules. The molecular mechanisms underlying these two microtubule dependent steps in cytokinesis are not known.

The degree to which the progression of cytokinesis depends on the central spindle varies somewhat in different experimental organisms. In invertebrate embryos, a transient interaction between astral microtubules of the mitotic spindle and the cell cortex is sufficient to position the cleavage furrow

(Rappaport, 1985). Furrows specified in this manner ingress, but if the spindle is removed, these furrows do not usually complete cytokinesis (Rappaport, 1985). In contrast, in *Drosophila* spermatocytes, contractile ring formation requires the central spindle (Giansanti et al., 1998). Similarly, in cultured mammalian cells, astral microtubules appear to be insufficient to induce furrow ingression, instead the presence or absence of a central spindle determines whether or not a cleavage furrow forms (Cao and Wang, 1996; Eckley et al., 1997; Rieder et al., 1997; Savoian et al., 1999; Wheatley and Wang, 1996). Moreover, in cultured cells, the central spindle is also required for completion of cytokinesis (Wheatley and Wang, 1996). In *C. elegans* embryos, as in invertebrate embryos, only the later stages of cytokinesis appear to depend on the central spindle. Embryos depleted of the kinesin-like protein ZEN-4/CeMKlp1 fail to assemble the central spindle, yet cleavage furrows form and ingress, but cytokinesis does not proceed to completion (Powers et al., 1998; Raich et al., 1998). In summary, the initiation of cytokinesis depends on the central spindle in some but not in all organisms, whereas there appears to be a general requirement for the central spindle for the completion of cytokinesis in animal cells. While it is clear that the central spindle plays an important role in cytokinesis the underlying mechanism remains elusive.

Cleavage furrow ingression is driven by the actin-based contractile ring. Like many actin-based structures, the contractile ring requires the RhoA GTPase for its assembly. Rho family GTPases are thought to act as

molecular switches that cycle between inactive GDP-bound forms and active GTP-bound forms; their ability to exchange and hydrolyze GTP is regulated by additional factors, the so-called guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Inactivation of RhoA by the exoenzyme C3 (Aktories and Hall, 1989), inhibits cytokinesis in a wide variety of experimental settings by causing disassembly of cortical actin structures and the contractile ring (Drechsel et al., 1997; Kishi et al., 1993; Mabuchi et al., 1993; Moorman et al., 1996; O'Connell et al., 1999). Further, a Rho GEF is essential for cytokinesis (Prokopenko et al., 1999; Tatsumoto et al., 1999). GTP-bound RhoA interacts with a number of putative effectors including formins, Rho Kinase, Citron kinase, and a regulatory subunit of myosin phosphatase (for review see (Van Aelst and D'Souza-Schorey, 1997). The requirement for RhoA in cytokinesis may reflect its ability to regulate formins since members of the formin gene family are required for cytokinesis in budding yeast (BNI1/BNR1) (Imamura et al., 1997), fission yeast (Cdc12) (Chang et al., 1997), *Drosophila* (*dia*) (Castrillon and Wasserman, 1994) and *C. elegans* (*cyk-1*) (Swan et al., 1998). Several formins also bind to profilin (Chang et al., 1997 ; Evangelista et al., 1997; Imamura et al., 1997; Watanabe et al., 1997), a key regulator of actin polymerization. It is conceivable that GTP-bound RhoA promotes contractile ring assembly by activating actin polymerization via the formins and by activating myosin motor activity.

There is compelling evidence that the microtubule-based central spindle and the actin-based cleavage furrow are both essential for cytokinesis. Nest, the question was asked how these two cytoskeletal polymers interact. There are a few cases in which an interaction between the microtubule and actin cytoskeletal systems have been characterized. Examples include nuclear positioning in budding yeast (Carminati and Stearns, 1997; Fujiwara et al., 1999; Miller et al., 1999), spindle orientation in epithelial cells (Busson et al., 1998) and in certain asymmetrically dividing cells, such as the posterior blastomere of the two cell *C. elegans* embryo (Gönczy et al., 1999a; Hyman and White, 1987; Skop and White, 1998; Waddle et al., 1994). In these examples there is evidence that the dynein/dynactin microtubule motor complex may mediate the interaction of microtubules with the cell cortex.

It was an object of the invention to elucidate the microtubule dependent steps in cytokinesis. In particular, it was sought to define the role of the central spindle in this process, in order to be able to interfere with this process and thus with cytokinesis, which provides a new approach for therapy, in particular cancer therapy.

Therefore, to solve the problem underlying the invention, it was investigated how the central spindle assembles and how it functions in cytokinesis.

In the course of the experiments, a novel gene, designated *cyk-4*, gene has been identified by genetic analysis in *C. elegans*. Embryos from *cyk-4(t1689ts)*

mutant hermaphrodites were shown to initiate, but to fail to complete, cytokinesis. These embryos also fail to assemble the central spindle. It was shown that the *cyk-4* gene encodes a GTPase activating protein (GAP) for Rho family of GTPases. The Cyk-4 GAP domain activates GTP hydrolysis by RhoA, Rac1 and Cdc42 *in vitro*. RNA-mediated interference of RhoA, Rac1 and Cdc42 suggests that Rho plays an essential role in cytokinesis and is the target of Cyk-4 GAP activity for cytokinesis. It could be shown that Cyk-4 and a Cyk-4:GFP fusion protein localize to the central spindle and persist at cell division remnants. It was found that Cyk-4 localization is dependent on the kinesin-like protein Zen-4/CeMKlp1 and vice-versa. The data obtained in the present invention suggest that Cyk-4 and Zen-4/CeMKlp1 cooperate in central spindle assembly. Central spindle localization of Cyk-4 could accelerate GTP hydrolysis by RhoA thereby allowing contractile ring disassembly and completion of cytokinesis.

In the present invention, the role of the *cyk-4* gene in the early divisions of the *C. elegans* embryo was characterized. It was shown that Cyk-4 is required for the late stages of cytokinesis. Interestingly, *cyk-4* mutant embryos fail to assemble the central spindle. Positional cloning and localization studies revealed that the *cyk-4* gene encodes a novel GTPase activating protein (GAP) for the Rho family of GTPases that localizes to the central spindle. The missense mutation in the *cyk-4(t1689ts)* allele is found in a domain dispensable for GAP activity suggesting that Cyk-4 may have another function in addition to

activating GTP hydrolysis by Rho family proteins. Accordingly, it was found that Cyk-4 and the kinesin-like protein Zen-4/CeMKlp1 are interdependent for their proper localization. Based on these data, a model is proposed by which Cyk-4 acts in concert with Zen-4/CeMKlp1 to assemble the central spindle. The concentration of Cyk-4 to the central spindle would then serve to target the GAP domain to the fully ingressed contractile ring where it could promote GTP hydrolysis by RhoA, thereby facilitating the completion of cytokinesis.

The division of a cell into two daughters requires dynamic interactions between the microtubule-based mitotic spindle and the actin-based contractile ring. In animal cells the position of the cleavage furrow, an actomyosin-based structure, is determined by the mitotic spindle in a manner that is poorly understood. In recent years is has become clear that the central spindle, an array of microtubule bundles that forms during anaphase, also plays an important role in cytokinesis. To gain insight into this fundamental cellular process, the cytokinesis-defective mutant, *cyk-4*, was analyzed. Cytological analysis reveals that *cyk-4* mutant embryos fail to assemble the central spindle. Though lacking a central spindle, *cyk-4* mutant embryos furrow extensively, but they fail to complete cytokinesis. The mutation responsible for the *cyk-4* phenotype was found to be a missense mutation in a gene encoding a Rho family GAP that, *in vitro*, stimulates GTP hydrolysis by Rho, Rac, and Cdc42. CYK-4 localizes to the central spindle and to cell division remnants. CYK-4 colocalizes with the ZEN-4/CeMKLP1

kinesin-like protein. Moreover, CYK-4 and ZEN-4/CeMKLP1 are interdependent for their localization. It is concluded that the CYK-4 GAP and the ZEN-4/CeMKLP1 kinesin-like protein cooperate to assemble the central spindle. Furthermore, it is proposed that a concentrated source of CYK-4 GAP on the central spindle could downregulate the RhoA GTPase and thereby promote the late stages of cytokinesis.

The findings of the present invention provide a model for CYK-4 dependent assembly of the central spindle:

In *cyk-4* mutant embryos the robust microtubule bundles that constitute the central spindle do not form. Instead, the spindle develops into two mitotic asters separated by a few overlapping, disorganized, microtubules. A similar phenotype is observed in *zen-4* mutant embryos (Powers et al., 1998; Raich et al., 1998). Thus both the ZEN-4/CeMKLP1 kinesin-like protein and the CYK-4 GAP are essential for this microtubule bundling. The *Drosophila* orthologue of ZEN-4/CeMKLP1 is also required for cytokinesis, though it seems to be required for all aspects of furrow ingression (Adams et al., 1998). Members of the MKLP1 subfamily of kinesin-like proteins have microtubule bundling activity *in vitro* (Kuriyama et al., 1994; Nislow et al., 1992). However, *in vivo*, ZEN-4 mediated microtubule bundling requires CYK-4.

In answering the question how CYK-4 and ZEN-4 could cooperate to assemble the central spindle, it is proposed that a complex containing multiple motor

proteins could specifically localize to overlapping, antiparallel microtubules (figure 10). If such a motor complex transits along a microtubule it might continue to an end and dissociate. However, if such a motor complex transited along a microtubule in the vicinity of an antiparallel microtubule, the complex might bind simultaneously to both microtubules and attempt to move alternately in opposite directions, the net result being that the complex would concentrate in the region of microtubule overlap. Since CYK-4 does not have a microtubule motor domain, yet it is essential for the formation of the central spindle, it is proposed that CYK-4 forms a complex with multiple ZEN-4 homodimers that localizes to and stabilizes overlapping antiparallel microtubules (figure 10).

It was of interest to find out if the RhoGAP activity of CYK-4 necessary to promote microtubule bundling by ZEN-4. It is proposed that central spindle assembly is unlikely to require CYK-4 GAP activity. This is suggested by two lines of evidence. First, central spindle assembly is defective in the *cyk-4(t1689ts)* allele that carries a missense mutation at amino acid 15. This substitution is distant from the C-terminal GAP domain, and *in vitro*, the amino terminus of CYK-4 is dispensable for GAP activity. Thus this allele would be predicted to retain catalytic activity *in vivo* and therefore GAP activity is not sufficient for central spindle assembly. Moreover, Rho RNAi experiments reveal that central spindle assembly is Rho independent, suggesting that Rho GAP activity is not required for this process. Thus CYK-4 may act to

promote central spindle assembly, independent of its GAP activity.

In the present invention, the function of the CYK-4 gap domain was analysed:

If CYK-4 function in central spindle assembly is independent of the Rho GTPase, it was of interest to determine the function of the CYK-4 GAP domain. CYK-4 is likely bifunctional, one function being to promote the assembly of the central spindle, the second function being to promote GTP hydrolysis by Rho family members. These two functions might be related in that the first function would serve to concentrate CYK-4 at a site where GAP activity is required. It may be assumed that CYK-4 GAP activity is required late in cytokinesis, to promote GTP hydrolysis by a Rho family GTPase whose downregulation causes disassembly of the contractile ring and cell separation (figure 10).

It was determined which GTPase might CYK-4 act on to promote cytokinesis. The CYK-4 GAP domain has all the hallmarks of a Rho family GAP and it may therefore be expected that it will act on this subfamily of the GTPase superfamily. Like many other RhoGAPs, the GAP domain of CYK-4 is promiscuous in its ability to promote GTP hydrolysis on Rho, Rac, and Cdc42 (Lamarche and Hall, 1994). The strongest piece of evidence that CYK-4 acts on Rho is based on the observation that of the GTPases tested, Rho is the only one that is clearly essential for cytokinesis. The requirement for Rho in cell division is well documented in a variety of experimental systems. To date there is no evidence that

Rac is required for cytokinesis and our data using RNAi to deplete Rac also failed to detect a role for this GTPase in this process. Moreover, it has been recently reported that *ced-10* mutants, which are defective in corpse engulfment subsequent to apoptosis and distal tip cell migration, contain mutations in the *rac* gene (Reddien and Horvitz, 2000). *ced-10* mutants do not have any gross phenotypes indicative of a role in cytokinesis. Moreover, Rac1 deficient mice are gastrulation defective, but the embryos do not contain multinucleate cells indicative of a cell division defect (Sugihara et al., 1998). The sum of these data argue that Rac is not an essential target of CYK-4 during cytokinesis. With regard to Cdc42, previous studies have implicated this GTPase in cytokinesis (Drechsel et al., 1997; Dutartre et al., 1996). Superficially, the weakly penetrant cytokinesis phenotype observed in *Cdc42(RNAi)* embryos is consistent with these earlier data. However the *Cdc42(RNAi)* embryos that are cytokinesis defective are also osmotically swollen and therefore cytokinesis defect may be indirect. Thus at this juncture it appears most likely that RhoA is the key substrate for the CYK-4 GAP activity.

Further support for the hypothesis that completion of cytokinesis requires downregulation of RhoA by CYK-4 would be supported by experiments in which the *cyk-4* phenotype is phenocopied by RhoA mutants that are hydrolysis defective. However the genetic tools necessary to express such dominant mutants in the early *C. elegans* embryo are currently unavailable. It is surprising that the CYK-4 GAP domain is less active

towards RhoA as compared to Rac or Cdc42, if indeed RhoA is the relevant target of its GAP activity. One possible explanation is that full length CYK-4 has a different activity profile as compared to the isolated GAP domain. A more interesting possibility is that CYK-4 localization is important for CYK-4 GAP activity. The phenotype of *cyk-4* mutant embryos suggests that CYK-4 needs to act when the contractile ring is in close proximity to the central spindle. Since CYK-4 is concentrated on the central spindle at this time, its high local concentration might overcome its lower activity towards RhoA.

In the present invention, the central spindle was shown to be at the center of cytokinesis:

There appear to be at least two microtubule-dependent steps in cytokinesis, contractile ring positioning and completion of cytokinesis. In some cells both processes are dependent on the central spindle. An important open question is whether these two reactions are mechanistically similar. While assembly of the contractile ring requires activation of RhoA, it was previously shown that the position of the contractile ring is specified in a RhoA-independent manner in *Xenopus* embryos (Drechsel et al., 1997). It has been shown here that a Rho GAP is required for the late stages of cytokinesis, suggesting that the second process, completion of cytokinesis, does involve RhoA. It is therefore assumed that the two microtubule-dependent steps in cytokinesis are distinct.

CYK-4 and ZEN-4 are not the only components of the central spindle, a number of other components, some of which are required for cytokinesis, are also present at this site. Polo kinase is known to associate with MKLP1 and to concentrate in the central spindle (Adams et al., 1998; Lee et al., 1995), and this kinase is essential for cytokinesis. Rho associated kinase also localizes to this site (Kosako et al., 1999). The AIR-2 aurora-like kinase localizes to the central spindle (Schumacher et al., 1998). This kinase seems to be required primarily for chromosome segregation (Woollard and Hodgkin, 1999), its direct involvement in cytokinesis requires further analysis. INCENP and the TD-60 antigen also localize to the central spindle and there is evidence that they may play a role in cytokinesis (Eckley et al., 1997; Mackay et al., 1998; Martineau-Thuillier et al., 1998; Savoian et al., 1999). Interestingly, a Rho GEF that is required for cytokinesis, ECT2, also accumulates on the central spindle (Tatsumoto et al., 1999), however the *Drosophila* ortholog, Pebble, does not localize in this manner (Prokopenko et al., 1999).

Further analysis of the specific functions of all of these cytokinesis regulators is the basis to determine which of these proteins are functionally interdependent as has been shown is the case for Cyk-4 and Zen-4/CeMKlp1.

In summary, the experiments of the present invention comprise an initial phenotypic, molecular, biochemical, and cell biological analysis of the *cyk-4* gene. These studies indicate that this protein is an active GTPase

activating protein that is required for cytokinesis, likely by its ability to regulate the RhoA GTPase. Quite surprisingly, one additional function of this protein is to promote assembly of the central spindle. Cyk-4 is a key molecule required for cytokinesis that regulates both the structure of the late mitotic spindle and the function of the contractile ring.

Thus, the present invention generally relates to Cyk-4 proteins, which have been shown to be essential for this second microtubule dependent step and thus defines a functional link between the central spindle and the contractile ring.

In a preferred embodiment, the invention relates to a mammalian Cyk-4 polypeptide, in particular murine and human Cyk-4.

In a first aspect, the invention relates to the murine Cyk-4 polypeptide with the amino acid sequence as set forth in SEQ ID NO:4 or with the amino acid sequence encoded by a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:3.

In a preferred aspect, the invention relates to the human Cyk-4 polypeptide with the amino acid sequence as set forth in SEQ ID NO:2 or with the amino acid sequence encoded by a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:1.

In a further aspect, the present invention relates to an isolated DNA molecule comprising a polynucleotide

with the nucleotide sequence as set forth in SEQ ID NO:1 encoding human Cyk-4 polypeptide, or an isolated DNA molecule encoding human Cyk-4 polypeptide comprising a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:1.

In a further aspect, the present invention relates to an isolated DNA molecule comprising a polynucleotide with the nucleotide sequence as set forth in SEQ ID NO:3 encoding murine Cyk-4 polypeptide, or an isolated DNA molecule encoding murine Cyk-4 polypeptide comprising a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:3.

By "stringent hybridization conditions" as used herein is meant overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (1X SSC = 150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C, or equivalent conditions.

In the following, if not otherwise stated, the term "Cyk-4" refers to both the murine and the human Cyk-4.

Homologues of the subject Cyk-4 proteins also include versions of the polypeptide which are resistant to post-translation modification or which alter the activity of the protein. The Cyk-4 polypeptide can comprise a full length protein, such as represented in SEQ ID NO:2 or 4, or it can comprise a fragment or variant therof.

Beside DNA molecules, the present invention relates to nucleic acid molecules in the form of RNA, such as mRNA. The DNA molecules include cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense (or plus) strand, or it may be the non-coding strand, also referred to as the antisense (or minus) strand. The present invention also relates to preparations of double stranded Cyk-4 RNA or derivatives thereof that can be used to interfere with gene expression by ds-RNA mediated gene interference as described by Fire et al. 1998 and reviewed by Fire, 1999; Bosher and Labouesse, 2000; Sharp, 1999.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. Recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells, and those DNA molecules purified (partially or substantially) from a solution whether produced by recombinant DNA or synthetic chemistry techniques. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. However, it is intended that "isolated" as used herein does not include the Cyk-4 cDNA present in a cDNA library or in a preparation of purified or isolated genomic DNA containing the *Cyk-4* gene or a portion thereof in admixture with one or more other cDNA molecules or DNA fragments.

The nucleic acid molecules of the present invention further include genetic constructs comprising one or more Cyk-4 DNA sequences operably linked to regulatory DNA sequences (which may be heterologous regulatory sequences), such as promoters or enhancers as described below, wherein upon expression of these DNA sequences in host cells, preferably in bacterial, fungal (including yeast), plant or animal (including insect or mammalian) cells, one or more *Cyk-4* polypeptides are produced. In such constructs, the regulatory sequences may be operably linked to a Cyk-4 polynucleotide encoding mature *Cyk-4* polypeptide or any of its variants, precursors, fragments or derivatives described herein, which may include one or more polynucleotides having a nucleic acid sequence that is complementary to substantially all or a portion of a nucleic acid molecule having a nucleic acid sequence as shown in SEQ ID NO:1 or 3. As used herein, the terms "a portion" or "a fragment" of a nucleic acid molecule or a polypeptide means a segment of a polynucleotide or a polypeptide comprising at least 15, and more preferably at least 20, contiguous nucleotides or amino acids of a reference polynucleotide or polypeptide (for example, the polynucleotide and polypeptide shown in SEQ ID NOs: 1, or 3, respectively, unless otherwise specifically defined below.)

Besides the DNA molecules having a nucleotide sequence corresponding to that depicted SEQ ID NO:1 or 3, the invention also relates to DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode the Cyk-4 mouse or human

polypeptides. Since the genetic code is well known in the art, it is routine for one of ordinary skill in the art to produce the degenerate variants described above without undue experimentation.

In addition, the invention relates to Cyk-4 polypeptides which have deviations from the sequence shown in SEQ ID NO:2 or 4, caused by the conservative exchange of amino acids, if they are Cyk-4 derivatives or fragments or peptides with the properties which are desirable for their use in therapy or in screening assays, or isolated DNA molecules encoding such derivatitives or fragments with a polynucleotide sequence varying in their sequence from SEQ ID NO:1 or 3.

Nucleic acid molecules of the present invention which encode a Cyk-4 polypeptide or a derivative or fragment thereof may include, but are not limited to, those encoding the amino acid sequence of the polypeptide by itself, together with additional, non-coding sequences, including for example introns and non-coding 5' and 3' sequences, such as the transcribed, untranslated regions (UTRs) or other 5' flanking sequences that may play a role in transcription (*e.g.*, via providing ribosome- or transcription factor-binding sites), mRNA processing (*e.g.* splicing and polyadenylation signals) and stability of mRNA; the coding sequence for the *Cyk-4* polypeptide operably linked to a regulatory DNA sequence, particularly a heterologous regulatory DNA sequence such as a promoter or enhancer; and the coding sequence for the *Cyk-4* polypeptide linked to one or more coding sequences which code for amino acids that

provide additional functionalities. Thus, the sequence encoding the polypeptide may be fused to a marker sequence, such as a sequence encoding a peptide which facilitates purification of the fused polypeptide. In certain embodiments of this aspect of the invention, the marker amino acid sequence may be a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described for instance in Gentz et al., 1989, hexa-histidine provides for convenient purification of the fusion protein. The "HA" tag is another peptide useful for purification which corresponds to an epitope derived from the influenza hemagglutinin protein, which has been described by Wilson *et al.*, 1984. Yet another useful marker peptide for facilitation of purification of *Cyk-4* is glutathione S-transferase (GST) encoded by the pGEX fusion vector (see, e.g., Winnacker, From Genes to Clones, New York: VCH Publishers, pp. 451-481 (1987)). As discussed below, other such fusion proteins include the *Cyk-4* fused to immunoglobulin Fc at the N- or C-terminus.

A still further aspect of the present invention relates to antibodies and antibody preparations specifically reactive with an epitope of the Cyk-4 polypeptide.

Polyclonal antibodies are conventionally obtained by immunising animals, particularly rabbits, by injecting the antigen or fragments thereof and subsequently purifying the immunoglobulin.

Monoclonal anti-Cyk-4-antibodies may be obtained by standard procedures following the principle described by Köhler and Milstein, 1975, by immunising animals, particularly mice, then immortalising antibody-producing cells from the immunised animals, e.g. by fusion with myeloma cells, and screening the supernatant of the hybridomas obtained by immunological standard assays for monoclonal anti- Cyk-4 -antibodies. For therapeutic or diagnostic use in humans, these animal antibodies may optionally be chimerised in the conventional way (Neuberger et al., 1984, Boulianne et al., 1984) or humanised (Riechmann et al., 1988, Graziano et al., 1995).

Cyk-4specific antibodies can be used for both diagnostic and screening applications.

Cyk-4 specific antibodies are useful in clinical situations, where determining overexpression or underexpression of CYK-4 has predictive value. Anti-CYK-4 antibodies can be used on tissue of cell specimens to evaluate the level of expression or changes in the subcellular localization in various disease states. In addition anti-CYK-4 antibodies can be used to detect the presence of CYK-4 in solid-phase binding assays as described in the subsequent subsection related to finding compounds that affect the interaction of CYK-4 with other proteins.

In a further aspect the present invention provides methods for identifying compounds capable of modulating, in particular inhibiting cytokinesis by

modulating, in particular inhibiting, the function of CYK-4 to promote GTP hydrolysis by Rho family GTPases.

This aspect of the invention is based on the finding that CYK-4 promotes GTP hydrolysis by Rho family GTPases. Due to the evolutionary conservation of the GTPase domain and the requirement for Rho in cytokinesis, it can be concluded that the ability of CYK-4 to promote GTP hydrolysis by Rho is crucial for CYK-4 function.

The assay may be conducted on the basis of the so-called "GTP hydrolysis assay", which is a biochemical assay carried out according to standard protocols, as described, *inter alia*, by Self et al., 1995, or by Settleman and Foster, 1995. By way of example, this assay may be carried out as follows: In a first step, the substrate is incubated with GTP that carries a radioactive label (e.g. the commercially available γ-^{32}P-GTP or an otherwise measurable, e.g. a fluorescent label, as described, *inter alia*, by Hazlett et al., 1993, under conditions and for a period of time sufficient to allow saturation of GTP binding sites. Subsequently, CYK-4 is added, optionally at various concentrations of from 0 to eg 1 uM; in the presence of absence of test compounds. After the appropriate time (e.g. 5 min.) the amount of GTP that has been hydrolysed is determined.

Compounds that specifically affect the rate of CYK-4-dependent GTP hydrolysis by Rho are drug candidates which may be further developed, e.g. in a first step, by structure-function analysis.

The assayof the invention may be performed in the high throughput format by automation of the reaction steps. In this case, a great number of compounds, e.g. from compound or natural product libraries, are applied to microtiter plates containing the reagents for the binding reaction. After the time required for GTP saturation in the control reaction (absence of test compound), the reaction solution may be filtered through a protein binding matrix, e.g. nitrocellulose, that is arranged in the same geometrical pattern as the original microtiter plate where the reaction took place, and the radioactivity retained in the filters, or the amount of liberated free phosphate (or other label as appropriate) is quantified.

To simplify the assay, the Rho protein may be immobilized on a solid matrix, either via a tag that allows for binding to a suitably modified solid support, e.g. by using a biotinylated Rho protein and a streptavidin-coated microtiter plate, the solid matrix may also be in the form of beads. In the above-described assays that measure GTP hydrolysis, the Rho protein is preferably human.

By way of example, the screening assay may be conducted as follows: A member of the Rho family GTPases is immobilized on a solid support, e.g. a SPA (proximity scintiallation assay bead) using either an epitope tag such as His6 or other similar tags. The Rho protein is loaded with radioactive ^{32}P-gamma-GTP in the presence of EDTA. Magnesium is added and the amount of label retained on the beads is measured by scintillation counting. Cyk-4 is added in the absence or presence of

test compounds, incubated for various times at which point the amount of radioactivity or other suitable label remaining associated with the beads measured. Other embodiments of the assay include using alternative labels such as fluorescent GTP or other radioisotopes and using alternative measures of GTP hydrolysis such as measuring the amount of free ^{32}P released, as described above.

Alternatively to using the full-length Rho protein, a truncated version may be used, as long the GTPase activity is maintained, e.g. a fragment that lacks the critical cysteine residue in the CaaX box at the C-terminus that is required for membrane targetting.

A number of Rho proteins are available that are suitable for use in a GTP hydrolysis assay as a substrate, they have been cloned from various species. Preferably, the are employed as recombinant proteins.

Examples for Rho substrates that may be employed are human RhoA (Swiss Prot Primary Accession Number P06749), human RhoB (Swiss Prot Primary Accession Number P01121), human RhoC (Swiss Prot Primary Accession Number P08134), human RAC1 (Swiss Prot Primary Accession Number P15154), human RAC2 (Swiss Prot Primary Accession Number P15153), human RAC3 (Swiss Prot Primary Accession Number O14658) and human GB25 (Swiss Prot Primary Accession Number P21181.

An example for a co-factor which may be present in the assay reaction is polo kinase, e.g. the murine (Acc. No. NP 035251) or the human (Acc. No. NP 005021) polo-like kinase homolog.

Based on the sequence information, the Rho substrates and the potentially present co-factor(s) can be produced recombinantly in suitable host cells, e.g. in E. coli or by means of baculovirus in insect cells, according to standard methods.

Compounds that have the ability to prevent CYK-4 stimulated GTP hydrolysis by Rho family GTPases are then tested for specificity in a similar assay format using other proteins that have Rho family GAP domains, e.g. p190 or RhoGAP (Lamarche and Hall, 1994).

According to another aspect, the screening method of the invention is based on the biochemical interaction of CYK-4 and members of the MKLP1 subfamily of the kinesin-like protein superfamily.

The function of CYK-4 in cytokinesis is intimately tied to its ability to interact with MKLP1, a kinesin like protein that is also required for central spindle assembly. Therefore compounds that inhibit this interaction are expected to prevent cytokinesis and to inhibit cell proliferation. In C.elegans extracts, CYK-4 and CeMKLP1 are present in a protein complex and can be co-immunoprecipitated. The nature of this protein interaction can be further characterized , in particular by determining whether the interaction is direct or whether it requires additional proteins. In addition, the protein domains required for this interaction can be mapped. Examples for members of the MLKP1 family are CeM03D4.1b (GenBank ID U61955, Protein ID 1397342) and HsMKLP1 (GenBank ID X67155; SwissProt Q02241).

The domains required for the interaction of Cyk-4 and MKLP1 can be identified using recombinant protein fragments of Cyk-4 and MKLP1. For example, agarose beads loaded with GST fusion proteins of Cyk-4 (or another fusion partner, e.g. maltose binding protein, etc.) are incubated with full-length or truncated fragments of MKLP1, the beads are sedimented and the ability of fragments of Cyk-4 to interact with MKLP1, or fragments thereof, are assessed by SDS-page. Alternatively, MKLP1 fragments can be immobilized and assessed for their ability to interact with full length Cyk-4 or fragments thereof. Alternatively, the yeast two hybrid assay can be used to measure the interaction of Cyk-4 protein fragments and MKLP1 protein fragments using standard techniques.

A minimal set of proteins necessary to form a stable complex, i.e. Cyk-4, MLKP1, and potentially additional protein co-factors are produced in recombinant form and a screening assay is conducted, preferably in the high throughput format, that measures the ability of test compounds to inhibit this interaction. Additional factors that may be required for the interaction can be identified in a biochemical complementation assay or a two hybrid assay. Since it is known that polo kinase forms a complex with Cyk-4, this protein is one of the potential co-factors required for the interaction with Cyk-4. In an embodiment of the invention, the domain of Cyk-4 that interacts with MKLP1, or the whole Cyk-4 protein, is immobilized on a solid support. The domain of MKLP1 that interacts with CYK-4 or the whole MLKP1 protein that is modified with a suitable label to allow for rapid detection (i.e. radiolabelled, fluorescently

labeled, hapten labelled etc.) is incubated, optionally in the presence of additional factors that may be required for the interaction, and in the presence or absence of the test compounds.

After an incubation period that allows for interaction of the proteins, the amount of MKLP1 bound to the immobilized CYK-4 is measured by use of the label outlined above or by the use of suitable antibodies in an ELISA type assay. The assay may also be setup in the reverse, e.g. with Cyk-4 being labeled or with MKLP1 immobilized, or by performing the binding reaction in solution and then capturing one of the components on a solid support and measuring the amount of the other component that is co-immobilized.

Due to their ability of inhibiting the function of Cyk-4 to promote GTP hydrolysis by Rho family GTPase or of interfering with the interaction between Cyk-4 and MKLP1, respectively, compounds identified in the above screens have the potential to perturb cytokinesis. In tumor cells, this effect may result in a decrease or a stop of tumor growth. In addition, inhibition of cytokinesis may cause the activation of a cell cycle arrest check point that will trigger apoptosis of the tumor cells.

To further evaluate the potential of the compounds as drugs, the candidate compounds can be assayed for their effect on cytokinesis and other cellular processes in tissue culture of normal or transformed cells. To test the inhibition of tumor cell proliferation, primary human tumor cells, are incubated with the compound

identified in the screen and the inhibition of tumor cell proliferation is tested by conventional methods, e.g. bromo-desoxy-uridine or ^{3}H thymidine incorporation.

Compounds that exhibit an anti-proliferative effect in these assays may be further tested in tumor animal models and used for the therapy of tumors.

Thus, in a further aspect, the invention relates to compounds identified in the above screens for the therapy of tumors and any other situation in which cell overproliferation is observed.

Description of the figures.

Figure 1:

cyk-4(t1689ts) mutant embryos fail to complete cytokinesis. Wildtype embryos (A) and embryos from *cyk-4* mutant hermaphrodites (B) were dissected from young adults, mounted on agarose pads and observed by time-lapse nomarski microscopy. The ingressing cleavage furrow is indicated (arrows).10 µm scale bars.

Figure 2:

cyk-4 mutant embryos produce deeply ingressing cleavage furrows, but do not form a prominent central spindle. Wild type (A, C) and *cyk-4* mutant embryos (B,D) were fixed and stained for actin (green) and DNA (blue)

(A,B); tubulin (green) and DNA (blue) (C,D). 10 µm scale bars.

Figure 3:

Positional cloning of the *cyk-4* locus. (A) A schematic of LGIII showing the positions of various loci and the extent of the deficiencies. *cyk-4* is uncovered by tDf10, tDf6, and ctDf2, but not ctDf3. (B) An enlargement of the physical map from the *unc-64* locus until the end of LGIII. The ability of various cosmid pools to rescue *cyk-4* is indicated. (C) A map of the predicted genes on K08E3. All the predicted genes except for K08E3.1 and K08E3.5 were inactivated by RNAi and only RNAi of K08E3.6 produced multinucleate embryos. (D) A schematic representation of the domain structure of CYK-4 and its human ortholog. The position of the point mutation identified in *cyk-4(t1689ts)* is indicated.

Figure 4:

cyk-4(RNAi) causes disorganization of the proximal gonad and affects formation of the central spindle. Young adults were injected with *cyk-4* dsRNA and the injected animals were analyzed 30 hours after injection (B). The gonad of an uninjected worm is shown for comparison (A). Wild-type embryos (C) and embryos from *cyk-4(RNAi)* injected animals (D) were fixed and stained for tubulin. 10 µm scale bars.

Figure 5:

CYK-4 enhances GTP hydrolysis by Rho, Rac, and Cdc42. The GTPases were preloaded with ^{32}P-α-GTP and then GST-CYK-4-GAP or GST was added at the indicated concentration. Samples were taken at two minute intervals, the labeled nucleotide was resolved by thin layer chromatography, the fraction of GTP and GDP was quantitated. This graph shows the fraction of GTP hydrolyzed at the 2 minute time point as a function of CYK-4 concentration. These data are representative of at least three independent experiments.

Figure 6:

rho(RNAi) causes cytokinesis defects and *cdc42(RNAi)* causes defects in spindle positioning. Young adults were injected with the indicated dsRNAs (see table 2) and the embryos produced by the injected worms were analyzed by time lapse nomarski microscopy. Images from a wild-type (A), *rho(RNAi)* (B) and a *cdc42(RNAi)* (C) embryo are shown. 10 µm scale bars.

Figure 7:

CYK-4 localizes to the central spindle and division remnants. (A-F) Wild-type embryos were fixed and stained for CYK-4 (green), tubulin (red) and DNA (blue). The localization of CYK-4 to the central spindle (arrow) in a one cell embryo (D) and a two cell embryo (F) is shown. The localization of CYK-4 to the

division remnant from the polar body (A), and between the AB and P1 blastomeres is indicated with arrows. (E). (G) An embryo from a line expressing CYK-4:GFP is stained with anti-GFP antibodies. The same structures are seen as with CYK-4 antibodies (arrow). (H) The intrinsic fluorescence of the gonad of a worm expressing CYK-4:GFP. CYK-4 is seen at the incomplete membranes of the syncytial gonad (arrow) and in oocyte nuclei. 10 µm scale bars.

Figure 8:

Time lapse analysis of CYK-4:GFP. An embryo from a line expressing CYK-4:GFP was imaged using low light level microscopy. The central spindle localization of CYK-4 is observed prior to furrow ingression. 10 µm scale bars.

Figure 9:

CYK-4 and ZEN-4/CeMKLP1 colocalize and are interdependent for their localization. An embryo expressing CYK-4:GFP stained for GFP (A), ZEN-4/CeMKLP1 (B) and the merged image (C). CYK-4 and ZEN-4/CeMKLP1 colocalize at division remnants (arrow) and a central spindle (arrowheads) structures. ZEN-4 localization to the central spindle is CYK-4 dependent (arrow). Wild-type (D) and *cyk-4(t1689ts)* embryos (E) were fixed and stained for ZEN-4/CeMKLP1 (green), tubulin (red) and DNA (blue). AIR-2 localization to the central spindle is CYK-4 independent (arrow). Wild-type (F) and

cyk-4(t1689ts) embryos (G) were fixed and stained for AIR-2 (green), tubulin (red) and DNA (blue). CYK-4 maintenance to division remnants (arrowheads) is ZEN-4/CeMKLP1 dependent. *zen-4(or153ts)* worms were maintained at 16 C and either fixed immediately (H) or shifted to 25 C for 18 minutes (I). Embryos were fixed and stained for CYK-4 (green), tubulin (red) and DNA (blue). 10 µm scale bars.

Figure 10:

Model for the function of CYK-4 in central spindle formation and cytokinesis.

In the Examples, if not otherwise stated, the following materials and methods were used:

a) Strains and alleles

The *cyk-4(t1689ts)* allele was identified in a search for maternal effect lethal mutations on chromosome III (see (Gönczy et al., 1999b) for details). The strains DR104, BW1535, BW1369, and RW7000 were obtained from the CGC. The strain EU699 containing the *zen-4(or153ts)* allele will be described in detail elsewhere (Aaron Severson, D.H. and Bruce Bowerman (U. of Oregon)). The end points of the deficiency tDf10 (Heinke Schnabel, unpublished data) are not molecularly defined, but it uncovers *cyk-4*, *lit-1*, and *bli-5* and it does not uncover *unc-64*.

b) Antisera

Cyk-4 specific antisera were produced in rabbits using a His6-Cyk-4 fusion protein as immunogen (containing amino acids 407-613 of cyk-4). A GST-Cyk-4 fusion containing amino acids 407-681 of Cyk-4 was coupled to a Hi-Trap NHS resin (Amersham-Phamacia) and used to affinity purify anti-Cyk-4 antibodies which were used at a final concentration of 1:300. The antibodies used for the studies are specific for Cyk-4 since the staining can be blocked with antigen, a similar pattern is observed when anti-GFP antibodies are used to detect a Cyk-4:GFP fusion construct, and the staining pattern is disrupted in cyk-4 mutant embryos. The rat monoclonal YOL 1/34 anti-tubulin antibody was used at a dilution of 1:200-500. Anti-GFP antibodies (Roche) were used at a dilution of 1:500. Antisera specific for Zen-4/MKlp1 was generously provided by Bill Saxton and Susan Strome (Univ. of Indiana) and used at a dilution of 1:4000. Antisera specific for Air-2 was generously provided by Andy Golden (NIH) and used at a dilution of 1:1000.

c) Genetic mapping of Cyk-4

The *cyk-4* locus maps under the deficiency tDf6 which deletes a large fraction of the distal right arm of LGIII. Recombination mapping using *unc-32(e189) cyk-4(t1689ts)/dpy-18(e364) unc-25(e156)* placed *cyk-4* distal to (or very close to) *unc-25* (23/23 Dpy non Unc's carried the *cyk-4* mutation). Recombination between *dpy-18 cyk-4*/RW7000 which carries several Tc1

elements including one on the cosmid F14F7 gave rise to 45 Dpy non-Ts animals, 3 of which lacked the TC1 insertion on F14F7, indicating that the *cyk-4* gene is distal to this cosmid. Crosses to strains carrying the deficiencies ctDf3, ctDf2, tDf10 revealed that *cyk-4(t1689ts)* is not uncovered by ctDf3 and is uncovered by both ctDf2 and tDf10. Since tDf10 does not uncover *unc-64*, *cyk-4(t1689ts)* must be distal to *unc-64*.

d) Time Lapse recordings

Time lapse nomarski imaging was performed as described previously (Jantsch-Plunger and Glotzer, 1999). Time lapse imaging of Cyk-4:GFP was performed on a Zeiss Axiovert microscope using a 100X/1.3 neofluar objective. The illumination source, an Atto-arc HBO-103, was reduced to 50% intensity. An intensified cooled CCD camera (GenIV pentamax, Princeton Instruments) was used for image acquisition. The camera and other electronics were controlled with MetaMorph software (Universal Imaging). Typical acquisition times were 40-80 msec. Each 10 seconds, 4-5 fluorescent images were acquired at different focal planes and a nomarski image was acquired. The fluorescent images were projected onto a single frame using the maximum intensity from the stack of images. Under these conditions, embryos could be filmed for more than one hour without affecting the cell cycle timing or pattern of cell divisions.

e) Rescue Experiments

To identify the *cyk-4* gene in this region cosmid DNA (from stocks kindly provided by Alan Coulson, Sanger Center) was coinjected with the *rol-6(su1006)* dominant marker (Mello et al., 1991) into the gonad of *unc-32 cyk-4/qC1* worms. Heterozygous F1 hermaphrodites that carried the rol-6 dominant marker were cloned to individual plates at 25°C and the presence of Unc progeny, indicating zygotic rescue of the *cyk-4* mutation, was assessed. Individual *unc-32, cyk-4* worms carrying the extrachromosomal arrays were cloned to individual plates to assess the extent of germline rescue. The *cyk-4* genomic rescue construct MP17, contains a 4.9 kb genomic XbaI fragment excised from K08E3 and inserted into pBS-KS+.

f) RNA interference

Approximately 500 bp of DNA corresponding to predicted coding regions of Rho (Y51H4A.B), Rac-1 (C09G12.8B), Cdc42 (R07G3.1), F22E12.2, Y32F6B.3, K08D3.9, K08E3.2, K08E3.3, K08E3.4, K08E3.6, K08E3.7, K08E3.8 were amplified by PCR and cloned into pGEM-T (Promega). Double stranded RNA was transcribed (Ambion) and annealed, and injected into the gonads of wildtype N2 hermaphrodites as described (Fire et al., 1998).

g) Production of Cyk-4:GFP transgenes

The GFP cassette from vector pPD119.16 (a generous gift from A. Fire) was excised with BspLUIII and inserted into the unique NcoI site of MP17 (see above). This construct was linearized with XbaI, and complex arrays containing linearized genomic DNA and linearized rol-6(su1006) DNA were mixed in a ratio of 1:100:1 and injected into *unc-32(e189) cyk-4(t1689ts)/qC1* hermaphrodites. Rolling F1 heterozygotes were singled out at 25°C and rolling Unc F2 animals were picked. A line MG110, was obtained that gave stable rescue of the *cyk-4(t1689ts)* mutation.

The structure of the *cyk-4* gene was established by analysis of a large number of EST sequences available in the sequence databases at the Sanger Center and the National Institute of Genetics and by sequencing the clones yk63D6 and yk104g12 (kindly provided by Yuji Kohara). The structure of the gene is identical to the structure predicted by the *C. elegans* genome consortium.

h) Immunolocalization

Immunolocalization studies were performed as previously described (Jantsch-Plunger and Glotzer, 1999). Briefly, gravid hermaphrodites were placed on aminoproyl-silane treated slides, a coverslip was added, and sufficient pressure to extrude the embryos was applied. The slide was placed into liquid nitrogen. The coverslip was removed while the sample

was still frozen, the preparation fixed with -20°C methanol and then antibody staining was performed according to standard procedures.

i) Biochemical analysis of Cyk-4

The coding regions of Rho, Rac, and Cdc42 were PCR amplified and cloned into pET28b with an C-terminal polyhistidine tag. The GTPases were expressed at 25°C and purified using Ni^{2+}-NTA-agarose (QIAGEN). Proteins were dialyzed into 50 mM Tris pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$ and quick frozen. The GAP domain of Cyk-4 (amino acids 407-681) was cloned into pGEX4T-1. Proteins were expressed at 25°C and purified using GSH-agarose (Sigma). Proteins were dialyzed into 50 mM Tris pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and quick frozen. To assess GAP activity, 15 pmol of the GTPases were loaded with 1 pmol ^{32}P-α-GTP in 20 mM Tris pH 7.6, 4 mM EDTA, 25 mM NaCl, 1 mM DTT, 1 mM ATP, 0.1 mg/ml BSA at room temperature. The sample was place on ice and $MgCl_2$ was added to 17 mM. GST-Cyk-4-GAP was added at the indicated concentrations and, at intervals, samples were taken by dilution into 2% SDS, 20 mM EDTA. Aliquots were spotted onto TLC plates (PEI-cellulose, Machery-Nagel) and developed in 1M LiCl. The plates were dried and exposed using a Storm Phosphoimager (Molecular Dynamics) and the data analysis was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health; http://rsb.info.nih.gov/nih-image/).

Example 1

cyk-4 mutants initiate, but do not complete, cytokinesis

The *cyk-4(t1689ts)* allele was isolated in a screen for maternal effect embryonic lethal mutations on chromosome III (Gönczy et al., 1999b). The cyk-4 locus is defined by a single, temperature sensitive (ts), allele. The phenotype of embryos derived from homozygous *cyk-4* hermaphrodites at the restrictive temperature (hereafter referred to as *cyk-4* mutant embryos) during the first division is shown in figure 1B. *cyk-4* mutant embryos appear normal until cytokinesis, except that defects in polar body extrusion are frequently observed (data not shown). The first cleavage furrow forms at the correct time and place, it ingresses extensively, but, invariably, it regresses. Quantitation of the extent of furrow ingression in *cyk-4* mutant embryos reveals that, on average, furrows ingress to 73+/-13% (n=14) of the egg diameter. A multipolar spindle develops in the second cell cycle and the process of furrow ingression and regression occurs again. This pattern is repeated until the embryos become grossly disorganized.

Although the majority of cell divisions in worm development occur early in embryogenesis, cells of the germline and and many cells in the nervous system are produced during post embryonic development (Sulston and Horvitz, 1977). A number of mutations in genes required for cell division cause worms to become sterile and uncoordinated (O'Connell et al., 1998; Woollard and

Hodgkin, 1999). To determine if *cyk-4* is required post embryonically, temperature shift experiments with worms homozygous for the *cyk-4(t1689ts)* allele were performed (table 1). Homozygous animals grown at 16°C are viable and fertile. Homozygous *cyk-4* animals shifted to 25°C at the L4 stage produce embryos with the cytokinesis defects described above. Homozygous animals shifted to 25°C at earlier stages either fail to hatch or become sterile and uncoordinated, depending on the time of the temperature shift. Thus cyk-4 is required postembryonically, perhaps due to its role in cell division. Unexpectedly, animals shifted at the L2/L3 stages become highly uncoordinated adults, even though most motor neurons would be expected to have completed all their divisions at the time of the shift. This may suggest that CYK-4 has additional roles besides its role in cell division. However, not out the possibility cannot be ruled out that some of the cells in the ventral nerve cord divide later in *cyk-4* mutants.

To investigate why *cyk-4* mutant embryos fail to complete cytokinesis, actin and tubulin were localized in wild-type and *cyk-4* mutant embryos. Both wild-type and mutant embryos contained deeply ingressing cleavage furrows that stain with an anti-actin antibody (figure 2A,B). The metaphase spindles of *cyk-4* mutant embryos appeared normal (data not shown). However, spindle morphology during early anaphase was significantly different in mutant embryos as compared to wild type. In wild-type embryos (20/20) prominent microtubule bundles form between the separating masses of chromatin, forming the central spindle (figure 2C).

In cyk-4 mutant embryos (9/10) such bundles were largely reduced and disorganized (figure 2D). It is concluded that CYK-4 is required for the organization of the central spindle during anaphase. Since the central spindle is required for cytokinesis, it is possible that the *cyk-4* mutant embryos fail to complete cytokinesis because they fail to assemble the central spindle.

Example 2

Cloning of the cyk-4 gene

To investigate the molecular basis for the phenotypes described above, it was first sought to map the *cyk-4* locus and clone the affected gene (see methods for details). The *cyk-4* gene maps distal to *unc-64* on the extreme right arm of LG III (figure 3). The *cyk-4* gene was then identified by functional rescue of the zygotic requirement for *cyk-4* using pools of cosmids. A pool of three cosmids (ZK520, W06F12, and K08E3) allowed *cyk-4* homozygotes to hatch and develop to adulthood at 25°C. These cosmids were injected individually and cosmid K08E3 contained rescuing activity. Further subcloning revealed that a 4.9 kb genomic fragment, predicted to contain the complete K08E3.6 gene and no other intact gene, could rescue the *cyk-4* zygotic and germline phenotypes. Finally, the coding region was amplified from DNA derived from *cyk-4(t1689)* homozygotes and sequenced and a single point mutation was identified that differed from the sequence provided by the genome project; this mutation was not observed in another line

derived from the same parental strain. These data show that the defect in *cyk-4(t1689ts)* embryos is due to a point mutation in the K08E3.6 gene.

The predicted protein product of the *cyk-4* gene has a C-terminal domain that contains the consensus motifs of GTPase activating proteins for Rho family GTPases (figure 3). Adjacent to the C-terminal GAP domain is a C1 domain that is predicted to bind to diacylglycerol or phorbol esters. At the amino terminus of the protein is a 90 amino acid region predicted to form a coiled-coil domain. The S15L point mutation found in *cyk-4(t1689ts)* , is located just amino terminal to the coiled-coil domain. Human and mouse proteins with structural similarities to *cyk-4* have been described (Toure et al., 1998; Wooltorton et al., 1999). These genes are expressed in a variety of proliferating tissues. In addition, the *Drosophila* sequence database contains an entry (ascension no. AC005977 (CLOT 94)) that, together with CYK-4 and the previously mentioned human gene, share a common structure consisting of approximately 650 amino acids, an NH_2-terminal coiled-coil domain and a conserved C-terminus containing C1 and GAP domains. The structural conservation of CYK-4 suggests that its function is conserved among metazoans.

In the present invention, the the human and mouse cDNA's were identified by searching the DNA databases with the C.elegans CYK-4 protein sequence.

Since the *cyk-4(t1689ts)* mutation may contain residual activity RNA-mediated interference (RNAi) was used to deplete embryos of CYK-4 protein. RNAi of the predicted open reading frame K08E3.6 generated multinucleate embryos which exhibited a similar phenotype to that of the *cyk-4* mutant, including loss of the central spindle (figure 4 D) and incomplete cytokinesis (not shown). Interestingly, the gonads of *cyk-4(RNAi)* animals become disorganized 30 hours post-injection (figure 4 B) and irregularly sized embryos are produced, suggesting that CYK-4 acts not only during embryonic and zygotic development, but also in the female germline.

Example 3

Biochemical activity of the Cyk-4 GAP domain

The presence of a Rho-family GAP domain suggests that CYK-4 may regulate one or more GTPases of Rho branch of the GTPase superfamily. To determine whether CYK-4 is active as a GAP and whether its GAP activity is restricted to particular members of the Rho subfamily, a recombinant fusion protein containing GST and the CYK-4 GAP domain was prepared and GTP hydrolysis assays were performed with recombinant *C. elegans* Rho, Rac, and Cdc42. The GAP domain of CYK-4 promotes GTP hydrolysis by all three tested GTPases (figure 5). However kinetic differences were observed. At the conditions used in the assays it was found that the CYK-4 GAP domain is more active towards Rac and Cdc42 than towards Rho. The human ortholog has a similar

activity profile *in vitro* (Toure et al., 1998). However, since CYK-4 has activity towards all three GTPases, these data are not sufficient to determine the *in vivo* target(s) of the CYK-4 GAP domain.

Next RNAi was used to determine which, if any, of the Rho family GTPases are required for cytokinesis in the *C. elegans* embryo. RNAi experiments were performed with RhoA, Rac1, Cdc42 and three additional GTPases found in the genome that fall into the Rho subfamily. Approximately 90% of *Rho(RNAi)* embryos exhibit cytokinesis defects in the first and/or second cell cycle (table 2 and figure 6). In most embryos, furrow ingression was inhibited. Interestingly, central spindles assemble in *RhoA(RNAi)* embryos (data not shown). In contrast, 88% of *Cdc42(RNAi)* embryos complete cytokinesis normally. However, a distinct defect in the early embryo is observed in 54% of*Cdc42(RNAi)* embryos; defects in spindle positioning are observed in P0 and/or P1. A minority (12%) of *Cdc42(RNAi)* embryos fail to initiate cytokinesis; in most cases, these embryos appear osmotically swollen even when provided with osmotic support. *Rac(RNAi)* embryos hatch with high efficiency and did not exhibit a detectable phenotype in the early embryo. RNA interference experiments with the additional GTPases either alone or in combinations did not reveal any additional defects in the early embryo. Thus RhoA is the only member of the Rho family that is clearly required for cytokinesis and is therefore likely to be the critical target for the CYK-4 GAP domain.

Example 4

The subcellular localization of Cyk-4

Next the subcellular localization of CYK-4 protein was determined. CYK-4 localization is cell cycle dependent (figure 7). In interphase cells, CYK-4 is present in the cytoplasm and slightly concentrated in the nucleus. CYK-4 is also highly concentrated in a spot at the anterior of the embryo, DNA labeling reveals that this localization corresponds to the site of polar body extrusion. As embryos enter mitosis, CYK-4 protein concentrates around the mitotic spindle. In early anaphase, CYK-4 concentrates to the central spindle. As the cleavage furrow ingresses, CYK-4 becomes highly concentrated on the central spindle into a structure that often appears ring shaped (not shown). Upon completion of cytokinesis, CYK-4 staining persists at division remnants. CYK-4 organized in ring-like structures averaging 1.2 μm in diameter are occasionally observed in the cytoplasm (not shown).

To determine if CYK-4 localizes to the central spindle prior to the onset of cleavage furrow ingression , the dynamics of CYK-4 localization in live embryos was investigated. To accomplish this goal a transgenic line expressing a CYK-4:GFP fusion was generated and its localization followed by time lapse microscopy. The CYK-4:GFP fusion is partially functional since *cyk-4 xsEx1[cyk-4:GFP]* animals are viable and fertile at 25°C whereas the parental *cyk-4* strain is inviable at 25°C. However, the fusion construct does not fully

rescue the mutation, since about 40% of embryos produced by this line fail to hatch (table 1).

Low light level fluorescence microscopy was used to visualize CYK-4:GFP in living embryos. Embryos were imaged using a multi-mode imaging system whereby a series of z-sections and a nomarski image were recorded every 10 seconds. The fluorescent images from each time point were projected to form a single image. These recordings reveal that CYK-4 accumulates on the central spindle prior to the initiation of furrowing (figure 8; 3:40). The CYK-4 that localizes to the central spindle becomes compressed into a bright spot which persists at the division remnant. The remnant persists for several cell cycles although instances were observed whereby the remnant (sometimes from the polar body) detaches from the cortex and is observed as a discrete spot in the cytoplasm. This detachment of CYK-4 from division remnants likely accounts for the CYK-4 rings seen in fixed specimens. It is concluded that CYK-4 localization on the central spindle precedes furrow ingression.

Example 5

Cyk-4 and zen-4/CeMklp1 are functionally interdependent

There are remarkable similarities between CYK-4 and the kinesin-like protein ZEN-4/CeMKLP1 (Powers et al., 1998; Raich et al., 1998). *zen-4* mutant embryos also initiate, but fail to complete cytokinesis. They also fail to assemble a robust central spindle in early

anaphase. Furthermore, ZEN-4 localizes to the central spindle and persists at division remnants after completion of cytokinesis. To test whether these proteins functionally interact, it was first assessed whether CYK-4 and ZEN-4/CeMKLP1 co-localize. Embryos expressing CYK-4:GFP were fixed and CYK-4 and ZEN-4 were localized simultaneously (using an anti-GFP antibody to detect CYK-4:GFP). The two proteins co-localize both on central spindle structures and on division remnants (Figure 9A-C). It was next investigated if ZEN-4 localization requires functional CYK-4 protein. ZEN-4 staining of *cyk-4* mutant embryos reveals that ZEN-4 localization to the central spindle is absent (figure 9E), although staining of some microtubule bundles in the spindle midzone could be detected using an AIR-2 antibody (figure 9G). Thus, recruitment of ZEN-4 to the central spindle is CYK-4 dependent. Next it was tested whether maintenance of ZEN-4 at division remnants requires functional CYK-4. *cyk-4* mutant embryos grown at 16°C were shifted to 25 °C for 15 minutes prior to fixation and staining with anti- ZEN-4 antibodies. The number of cells and number of division remnants labeled with the anti-ZEN-4 antibody were counted. Embryos maintained at the permissive temperature had a large number of ZEN-4 staining division remnants (table 3) whereas the embryos shifted to the non-permissive temperature lacked defined staining of division remnants. Thus both recruitment of ZEN-4 to the central spindle and its maintenance at division remnants is CYK-4 dependent. The reverse experiment was conducted with a temperature sensitive allele of *zen-4(or153ts)*. In

this case CYK-4 staining at division remnants was observed in *zen-4* mutant embryos at the permissive temperature but this staining disappeared upon a brief shift to the non-permissive temperature (figure 9H,I and table 3). Thus maintenance of CYK-4 at division remnants is ZEN-4 dependent. It is concluded that CYK-4 and ZEN-4/CeMKLP1 colocalize and that the two proteins are interdependent for their localization.

Next, it was determined if embryos carrying mutations in both *cyk-4* and *zen-4* are distinguishable from the single mutants. Two strains were built, one strain was homozygous for *zen-4(or153ts)* and heterozygous for *cyk-4(t1689ts)* and the second strain was homozygous for *cyk-4(t1689ts)* and heterozygous for *zen-4(or153ts)*. Both strains were viable at 16°C, but they failed to produce doubly homozygous larvae. It was found that worms of genotype *unc-32(e189) cyk-4(t1689ts) /qC1* III, *zen-4(or153ts)* laid a fraction of embryos that arrested during embryonic development, typically before the comma stage. Thus *cyk-4(t1689ts)* and *zen-4(or153ts)* are synthetically lethal.

Table 1

genotype	stage at time of shift to 25°C	percent viable	phenotype of surviving progeny	N
N2 (wild type)	embryos	98.8%	wild-type	485
cyk-4	(not shifted)	70.2%	fertile	198
cyk-4	embryos	1.5%	sterile, highly Unc	324
cyk-4	L1 larvae	100.0%	sterile, highly Unc	89
cyk-4	L2/L3	100.0%	sterile, highly Unc	55
cyk-4	L4	100.0%	fertile, lay dead embryos	52
cyk-4, xsEx1[cyk-4:GFP]	embryos	59.4%	fertile	350

Except for the N2 control, the complete genotype of the strain was *unc-32(e189) cyk-4(t1689ts)*. Gravid hermaphrodites were allowed to lay embryos for 2 hours at the permissive temperature. The adult was then removed and the number of embryos counted. After 24 hours at the indicated temperature the number of unhatched embryos was then counted.

Table 2

injected dsRNA	locus	percent cytokinesis defective (N)	number of time lapse recordings	number cytokinesis defective	additional phenotypes
RhoA	Y51H4A.B	95% (175)	15	15 (100%)	
Rac-1	C09G12.8 B	Not emb. lethal	5	0	
Cdc42	R07G3.1	12% (74)	22	3 (14%)	symmetric 1st division (5/22); no rotation in P1(12/22); rotation of AB (2/22)
others	F22E12.2	Not emb. lethal			
	Y32F6B.3	Not emb. lethal			
	K03D3.9	Not emb. lethal			

Young adult hermaphrodites were injected with the indicated dsRNAs and broods of laid embryos were scored for embryonic lethality. dsRNAs that induced embryonic lethality were further characterized by dissecting embryos and scoring for multinucleate embryos and by performing time lapse recordings and evaluating cytokinesis, spindle orientation in the one-cell embryo (symmetric or asymmetric first cleavage), and spindle positioning in the P1 and AB blastomeres.

Table 3

genotype	temperature	total number of stained remnants	total number of cells	average number of remnants/ cell	number of embryos
N2 (wildtype)	25°C	110	137	0.80	16
zen-4(or198ts)	16°C	61	83	0.73	10
zen-4(or198ts)	25°C (18 min.)	11	147	0.07	24
N2 (wildtype)	25°C	150	205	0.73	22
cyk-4(t1689ts)	16°C	61	98	0.62	10
cyk-4(t1689ts)	25°C (15 min.)	10	176	0.06	19

Wild-type and *zen-4(or153ts)* embryos grown at the indicated temperatures were fixed and stained for Cyk-4. Similarly, Wild-type and *cyk-4(t1689ts)* grown at the indicated temperatures were fixed and stained forZen-4/CeMKlp1.The number of cells in each embryo and the number of remnants staining with Cyk-4 or Zen-4 antibodies was counted.

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Lys Val Val Glu Arg Leu Leu Ser Leu Pro Leu Glu Tyr Trp Ser Gln
                530                 535                 540

ttc atg atg gtg gag caa gag aac att gac ccc cta cat gtc att gaa   1741
Phe Met Met Val Glu Gln Glu Asn Ile Asp Pro Leu His Val Ile Glu
            545                 550                 555

aac tca aat gcc ttt tca aca cca cag aca cca gat att aaa gtg agt   1789
Asn Ser Asn Ala Phe Ser Thr Pro Gln Thr Pro Asp Ile Lys Val Ser
        560                 565                 570

tta ctg gga cct gtg acc act cct gaa cat cag ctt ctc aag act cct   1837
Leu Leu Gly Pro Val Thr Thr Pro Glu His Gln Leu Leu Lys Thr Pro
    575                 580                 585

tca tct agt tcc ctg tca cag aga gtc cgt tcc acc ctc acc aag aac   1885
Ser Ser Ser Ser Leu Ser Gln Arg Val Arg Ser Thr Leu Thr Lys Asn
590                 595                 600                 605

act cct aga ttt ggg agc aaa agc aag tct gcc act aac cta gga cga   1933
Thr Pro Arg Phe Gly Ser Lys Ser Lys Ser Ala Thr Asn Leu Gly Arg
                610                 615                 620

caa ggc aac ttt ttt gct tct cca atg ctc aag tga agtcacatct        1979
Gln Gly Asn Phe Phe Ala Ser Pro Met Leu Lys
            625                 630

gcctgttact tcccagcatt gactgactat aagaaaggac acatctgtac tctgctctgc 2039

agcctcctgt actcattact acttttagca ttctccaggc ttttactcaa gtttaattgt 2099

gcatgagggt tttattaaaa ctatatatat ctccccttcc ttctcctcaa gtcacataat 2159

atcagcactt tgtgctggtc attgttggga gcttttagat gagacatctt tccaggggta 2219

gaagggttag tatggaattg gttgtgattc tttttgggga agggggttat tgttcctttg 2279

gcttaaagcc aaatgctgct catagaatga tctttctcta gtttcattta gaactgattt 2339

ccgtgagaca atgacagaaa ccctacctat ctgataagat tagcttgtct cagggtggga 2399

agtgggaggg cagggcaaag aaaggattag accagaggat ttaggatgcc tccttctaag 2459

aaccagaagt tctcattccc cattatgaac tgagctataa tatggagctt tcataaaaat 2519

gggatgcatt gaggacagaa ctagtgatgg gagtatgcgt agctttgatt tggatgatta 2579

ggtctttaat agtgttgagt ggcacaacct tgtaaatgtg aaagtacaac tcgtatttat 2639

ctctgatgtg ccgctggctg aactttgggt tcatttgggg tcaaagccag tttttctttt 2699

aaaattgaat tcattctgat gcttggcccc catacccccа accttgtcca gtggagccca 2759
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acttctaaag gtcaatatat catcctttgg catcccaact accaataaag agtaggctat 2819

aagggaagat tgtcaatatt ttgtggtaag aaaagctaca gtcatttttt ctttgcactt 2879

tggatgctga aatttttccc atggaacata gccacatcta gatagatgtg agctttttct 2939

tctgttaaaa ttattcttaa tgtctgtaaa aacgattttc ttctgtagaa tgtttgactt 2999

cgtattgacc cttatctgta aaacacctat ttgggataat atttggcttt a 3050

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<212> PRT
<213> Homo sapiens

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Met Asp Thr Met Met Leu Asn Val Arg Asn Leu Phe Glu Gln Leu Val
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Arg Arg Val Glu Ile Leu Ser Glu Gly Asn Glu Val Gln Phe Ile Gln
20 25 30

Leu Ala Lys Asp Phe Glu Asp Phe Arg Lys Lys Trp Gln Arg Thr Asp
35 40 45

His Glu Leu Gly Lys Tyr Lys Asp Leu Leu Met Lys Ala Glu Thr Glu
50 55 60

Arg Ser Ala Leu Asp Val Lys Leu Lys His Ala Arg Asn Gln Val Asp
65 70 75 80

Val Glu Ile Lys Arg Arg Gln Arg Ala Glu Ala Asp Cys Glu Lys Leu
85 90 95

Glu Arg Gln Ile Gln Leu Ile Arg Glu Met Leu Met Cys Asp Thr Ser
100 105 110

Gly Ser Ile Gln Leu Ser Glu Glu Gln Lys Ser Ala Leu Ala Phe Leu
115 120 125

Asn Arg Gly Gln Pro Ser Ser Ser Asn Ala Gly Asn Lys Arg Leu Ser
130 135 140

Thr Ile Asp Glu Ser Gly Ser Ile Leu Ser Asp Ile Ser Phe Asp Lys
145 150 155 160

Thr Asp Glu Ser Leu Asp Trp Asp Ser Ser Leu Val Lys Thr Phe Lys
165 170 175

Leu Lys Lys Arg Glu Lys Arg Arg Ser Thr Ser Arg Gln Phe Val Asp
180 185 190

Gly Pro Pro Gly Pro Val Lys Lys Thr Arg Ser Ile Gly Ser Ala Val
195 200 205

Asp Gln Gly Asn Glu Ser Ile Val Ala Lys Thr Thr Val Thr Val Pro
210 215 220

Asn Asp Gly Gly Pro Ile Glu Ala Val Ser Thr Ile Glu Thr Val Pro
225 230 235 240

Tyr Trp Thr Arg Ser Arg Arg Lys Thr Gly Thr Leu Gln Pro Trp Asn
245 250 255

Ser Asp Ser Thr Leu Asn Ser Arg Gln Leu Glu Pro Arg Thr Glu Thr
260 265 270

Asp Ser Val Gly Thr Pro Gln Ser Asn Gly Gly Met Arg Leu His Asp
275 280 285

Phe Val Ser Lys Thr Val Ile Lys Pro Glu Ser Cys Val Pro Cys Gly
290 295 300

Lys Arg Ile Lys Phe Gly Lys Leu Ser Leu Lys Cys Arg Asp Cys Arg
305 310 315 320

Val Val Ser His Pro Glu Cys Arg Asp Arg Cys Pro Leu Pro Cys Ile
325 330 335

Pro Thr Leu Ile Gly Thr Pro Val Lys Ile Gly Glu Gly Met Leu Ala
340 345 350

Asp Phe Val Ser Gln Thr Ser Pro Met Ile Pro Ser Ile Val Val His
355 360 365

Cys Val Asn Glu Ile Glu Gln Arg Gly Leu Thr Glu Thr Gly Leu Tyr
370 375 380

Arg Ile Ser Gly Cys Asp Arg Thr Val Lys Glu Leu Lys Glu Lys Phe
385 390 395 400

Leu Arg Val Lys Thr Val Pro Leu Leu Ser Lys Val Asp Asp Ile His
405 410 415

Ala Ile Cys Ser Leu Leu Lys Asp Phe Leu Arg Asn Leu Lys Glu Pro
420 425 430

Leu Leu Thr Phe Arg Leu Asn Arg Ala Phe Met Glu Ala Ala Glu Ile
435 440 445

Thr Asp Glu Asp Asn Ser Ile Ala Ala Met Tyr Gln Ala Val Gly Glu
450 455 460

Leu Pro Gln Ala Asn Arg Asp Thr Leu Ala Phe Leu Met Ile His Leu
465 470 475 480

Gln Arg Val Ala Gln Ser Pro His Thr Lys Met Asp Val Ala Asn Leu
485 490 495

Ala Lys Val Phe Gly Pro Thr Ile Val Ala His Ala Val Pro Asn Pro
500 505 510

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Asp Pro Val Thr Met Ser Gln Asp Ile Lys Arg Gln Pro Lys Val Val
        515                 520                 525

Glu Arg Leu Leu Ser Leu Pro Leu Glu Tyr Trp Ser Gln Phe Met Met
    530                 535                 540

Val Glu Gln Glu Asn Ile Asp Pro Leu His Val Ile Glu Asn Ser Asn
545                 550                 555                 560

Ala Phe Ser Thr Pro Gln Thr Pro Asp Ile Lys Val Ser Leu Leu Gly
                565                 570                 575

Pro Val Thr Thr Pro Glu His Gln Leu Leu Lys Thr Pro Ser Ser Ser
            580                 585                 590

Ser Leu Ser Gln Arg Val Arg Ser Thr Leu Thr Lys Asn Thr Pro Arg
        595                 600                 605

Phe Gly Ser Lys Ser Lys Ser Ala Thr Asn Leu Gly Arg Gln Gly Asn
    610                 615                 620

Phe Phe Ala Ser Pro Met Leu Lys
625                 630
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<222> (2022)..(2919)

<400> 3

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gggcgcggcg gccggagagt gagcgccgcg ggccggacgg ggttgcgtgt gcgcggggcc 120

gggagcctcg aaag atg gat act aca atg gtg aat ttg tgg act ctg ttt   170
               Met Asp Thr Thr Met Val Asn Leu Trp Thr Leu Phe
                 1               5                  10

gag cag ctt gtg cgc cgg atg gag att atc aat gaa gga aat gaa agc   218
Glu Gln Leu Val Arg Arg Met Glu Ile Ile Asn Glu Gly Asn Glu Ser
         15                  20                  25
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att gaa ttc atc cag gtt gtg aag gac ttc gag gac ttc cga aag aag   266
Ile Glu Phe Ile Gln Val Val Lys Asp Phe Glu Asp Phe Arg Lys Lys
     30                  35                  40

tat caa aga acc aac cag gag ctg gag aaa ttc aaa gac cta ttg ttg   314
Tyr Gln Arg Thr Asn Gln Glu Leu Glu Lys Phe Lys Asp Leu Leu Leu
 45                  50                  55                  60

aaa gca gag act ggg cgg agc gcc ctg gac gtg aag ctg aag cat gcc   362
Lys Ala Glu Thr Gly Arg Ser Ala Leu Asp Val Lys Leu Lys His Ala
                 65                  70                  75

cgt aat caa gtg gac gtg gag atc aag cgg agg cag cgc gct gag gca   410
Arg Asn Gln Val Asp Val Glu Ile Lys Arg Arg Gln Arg Ala Glu Ala
             80                  85                  90

gag tgt gca aag ctg gaa caa cag att cag ctg att cga gac ata ctc   458
Glu Cys Ala Lys Leu Glu Gln Gln Ile Gln Leu Ile Arg Asp Ile Leu
         95                 100                 105

atg tgt gac aca tct ggc agt att cag ctg agt gag gaa caa aaa tca   506
Met Cys Asp Thr Ser Gly Ser Ile Gln Leu Ser Glu Glu Gln Lys Ser
    110                 115                 120

gct ctc gct ttc ctc aac cga ggc caa gca tcc agt ggc cac gcc ggc   554
Ala Leu Ala Phe Leu Asn Arg Gly Gln Ala Ser Ser Gly His Ala Gly
125                 130                 135                 140

aac aat aga ctg tca acg att gat gaa tct ggt tcc att tta tca gat   602
Asn Asn Arg Leu Ser Thr Ile Asp Glu Ser Gly Ser Ile Leu Ser Asp
                145                 150                 155

atc agc ttt gac aag act gat gaa tca ctg gac tgg gat tct tct ttg   650
Ile Ser Phe Asp Lys Thr Asp Glu Ser Leu Asp Trp Asp Ser Ser Leu
            160                 165                 170

gtg aag aat ttc aaa atg aag aaa cga gag aag agg cgc tcc aac agt   698
Val Lys Asn Phe Lys Met Lys Lys Arg Glu Lys Arg Arg Ser Asn Ser
        175                 180                 185

aga cag ttc atc gac ggc cct cct ggg cct gtg aag aaa act tgt tcc   746
Arg Gln Phe Ile Asp Gly Pro Pro Gly Pro Val Lys Lys Thr Cys Ser
    190                 195                 200

att ggc tct aca gta gac cag gca aat gaa tca ata gtt gca aaa act   794
Ile Gly Ser Thr Val Asp Gln Ala Asn Glu Ser Ile Val Ala Lys Thr
205                 210                 215                 220

aca gtg act gtt ccc agt gat ggg gga ccc att gaa gct gtg tct act   842
Thr Val Thr Val Pro Ser Asp Gly Gly Pro Ile Glu Ala Val Ser Thr
                225                 230                 235

att gag aca ttg ccg tcc tgg acc agg agt cgc ggg aag tca gga cct   890
Ile Glu Thr Leu Pro Ser Trp Thr Arg Ser Arg Gly Lys Ser Gly Pro
            240                 245                 250
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tta caa cct gtg aac agt gac tcc gct ctg aac agc agg cca ctg gag   938
Leu Gln Pro Val Asn Ser Asp Ser Ala Leu Asn Ser Arg Pro Leu Glu
        255                 260                 265

cca aga act gac aca gac aat ttg ggc aca cct cag aat act gga ggc   986
Pro Arg Thr Asp Thr Asp Asn Leu Gly Thr Pro Gln Asn Thr Gly Gly
    270                 275                 280

atg cgc ttg cac gac ttc gtc tca aag acg gtt att aag cct gaa tct   1034
Met Arg Leu His Asp Phe Val Ser Lys Thr Val Ile Lys Pro Glu Ser
285                 290                 295                 300

tgt gtt ccg tgt gga aag cgg atc aag ttt ggc aag ctg tct ctg aag   1082
Cys Val Pro Cys Gly Lys Arg Ile Lys Phe Gly Lys Leu Ser Leu Lys
                305                 310                 315

tgt cga gac tgt cgt ttg gtc tcc cat cca gaa tgt cgg gac cga tgt   1130
Cys Arg Asp Cys Arg Leu Val Ser His Pro Glu Cys Arg Asp Arg Cys
            320                 325                 330

ccc ctt ccc tgc atc ccc ccc ctg gtg ggg aca ccg gtt aag att gga   1178
Pro Leu Pro Cys Ile Pro Pro Leu Val Gly Thr Pro Val Lys Ile Gly
        335                 340                 345

gag ggc atg ctg gcc gac ttc gtg tcg cag gct tct ccc atg atc cct   1226
Glu Gly Met Leu Ala Asp Phe Val Ser Gln Ala Ser Pro Met Ile Pro
    350                 355                 360

gcc att gtc gtc agc tgt gtc aat gag atc gag cag cga ggc ctg act   1274
Ala Ile Val Val Ser Cys Val Asn Glu Ile Glu Gln Arg Gly Leu Thr
365                 370                 375                 380

gag gca ggc ttg tac agg atc tca ggc tgt gac cgc aca gtg aaa gaa   1322
Glu Ala Gly Leu Tyr Arg Ile Ser Gly Cys Asp Arg Thr Val Lys Glu
                385                 390                 395

ctg aaa gaa aaa ttc ctt aag gtg aaa act gtg ccc ctc ctc agc aaa   1370
Leu Lys Glu Lys Phe Leu Lys Val Lys Thr Val Pro Leu Leu Ser Lys
            400                 405                 410

gtg gac gat atc cac gtc atc tgc agc ctc ctg aag gac ttc ctg cgc   1418
Val Asp Asp Ile His Val Ile Cys Ser Leu Leu Lys Asp Phe Leu Arg
        415                 420                 425

aac ctc aaa gag ccc ctc ctg acc ttc tgg ctg agc aaa gcc ttc atg   1466
Asn Leu Lys Glu Pro Leu Leu Thr Phe Trp Leu Ser Lys Ala Phe Met
    430                 435                 440

gag gca gca gag ata aca gat gaa gac aac agc aca gcc gcc atg tac   1514
Glu Ala Ala Glu Ile Thr Asp Glu Asp Asn Ser Thr Ala Ala Met Tyr
445                 450                 455                 460

cag gct gtc agt gag ctg ccc cag gcc aac agg gac acg cta gcc ttc   1562
Gln Ala Val Ser Glu Leu Pro Gln Ala Asn Arg Asp Thr Leu Ala Phe
                465                 470                 475
```

```
ctt atg atc cac cta cag aga gtg tct cag agt cca gac act aag atg   1610
Leu Met Ile His Leu Gln Arg Val Ser Gln Ser Pro Asp Thr Lys Met
            480                 485                 490

gat att gcc aat cta gct aaa gtc ttt ggc cct aca ata gtt gcc cat   1658
Asp Ile Ala Asn Leu Ala Lys Val Phe Gly Pro Thr Ile Val Ala His
        495                 500                 505

act gtg ccc aat cca gat cca gtg aca atg ttc cag gac atc aaa cgt   1706
Thr Val Pro Asn Pro Asp Pro Val Thr Met Phe Gln Asp Ile Lys Arg
    510                 515                 520

cag ctc aag gtg gtg gag cgc cta ctc tct ctc ccc ttg gag tac tgg   1754
Gln Leu Lys Val Val Glu Arg Leu Leu Ser Leu Pro Leu Glu Tyr Trp
525                 530                 535                 540

aat cag ttc atg atg gtg gac caa gag aac ata gac agc cag cga ggc   1802
Asn Gln Phe Met Met Val Asp Gln Glu Asn Ile Asp Ser Gln Arg Gly
                545                 550                 555

aat gga aac tca aca cca cgc aca cca gac gtt aaa gtg agc tta ctg   1850
Asn Gly Asn Ser Thr Pro Arg Thr Pro Asp Val Lys Val Ser Leu Leu
            560                 565                 570

ggg cct gtg acc act cct gaa ttc cag ctt gtc aag act cct tta tca   1898
Gly Pro Val Thr Thr Pro Glu Phe Gln Leu Val Lys Thr Pro Leu Ser
        575                 580                 585

agt tcc ctg tca cag agg ttg tac aac ctc tcc aag agc aca ccc aga   1946
Ser Ser Leu Ser Gln Arg Leu Tyr Asn Leu Ser Lys Ser Thr Pro Arg
    590                 595                 600

ttt ggg aac aag agc aag tct gcc acc aac tta ggt caa cag ggc aaa   1994
Phe Gly Asn Lys Ser Lys Ser Ala Thr Asn Leu Gly Gln Gln Gly Lys
605                 610                 615                 620

ttt ttc cct gct ccg tac ctc aag taa agctgtgtct gcctgtgttt         2041
Phe Phe Pro Ala Pro Tyr Leu Lys
                625

actgcacgag acacccctgt ctgctcttca gcctcctgtg taatgactac ttttagcatt 2101

ttccagactt taaataaagt tgaacgcgta tgagagtttg agtgttgcat agctccctcc 2161

cacctggaca cttagcacct ttactagttg tcgggagctt taaaatagga gatctttacc 2221

agggccgaag gggaaatggg tataagttgg ggctcttagg gggaggggag aggagatccc 2281

tttggcttaa agccaaatac tgctcatgaa atgactttgc tgtggtgtca cttagacaat 2341

gacagaaact gtacctctta ggtaacgtct tagttatctc aggacaggat aaagagctgc 2401

gcagcggccc cttctcagaa cacaggcctc ttcctccttc ggggactgac aagaagcttg 2461

gagccctcag cgggatgggc cgggccactg atctgaggat gcttcacttc tgtctgggtt 2521

aagctgagaa agtgccacag gctctgatct cacacagcgt gctgtttcta gccagcctct 2581
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gcctggattt tgcagtcaaa gtcaggttga tctgaagccg aattcgttct gatgcctgac 2641

ccccttgttt gtccagtgga gcccatttaa aaagtcagct agcagttcag aagacaattt 2701

tcatatagca agaaagttag ctgcttctgt ctttggtgaa gtttttctgt gaaacagatc 2761

tatatatgtg aacattggct tttttggtta gcattttcca gtgttcaaaa tggcttcctt 2821

ccctgggatg ttttctgacc catactaacc cttacctgta acatgtatct ggaatattat 2881

gtggaaaaaa taaatagctt tttcaaagtg aactttcc 2919

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<212> PRT
<213> Mus musculus

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  1               5                  10                  15

Arg Arg Met Glu Ile Ile Asn Glu Gly Asn Glu Ser Ile Glu Phe Ile
             20                  25                  30

Gln Val Val Lys Asp Phe Glu Asp Phe Arg Lys Lys Tyr Gln Arg Thr
         35                  40                  45

Asn Gln Glu Leu Glu Lys Phe Lys Asp Leu Leu Leu Lys Ala Glu Thr
     50                  55                  60

Gly Arg Ser Ala Leu Asp Val Lys Leu Lys His Ala Arg Asn Gln Val
 65                  70                  75                  80

Asp Val Glu Ile Lys Arg Arg Gln Arg Ala Glu Ala Glu Cys Ala Lys
                 85                  90                  95

Leu Glu Gln Gln Ile Gln Leu Ile Arg Asp Ile Leu Met Cys Asp Thr
            100                 105                 110

Ser Gly Ser Ile Gln Leu Ser Glu Glu Gln Lys Ser Ala Leu Ala Phe
        115                 120                 125

Leu Asn Arg Gly Gln Ala Ser Ser Gly His Ala Gly Asn Asn Arg Leu
    130                 135                 140

Ser Thr Ile Asp Glu Ser Gly Ser Ile Leu Ser Asp Ile Ser Phe Asp
145                 150                 155                 160

Lys Thr Asp Glu Ser Leu Asp Trp Asp Ser Ser Leu Val Lys Asn Phe
                165                 170                 175

Lys Met Lys Lys Arg Glu Lys Arg Arg Ser Asn Ser Arg Gln Phe Ile
            180                 185                 190
```

```
Asp Gly Pro Pro Gly Pro Val Lys Lys Thr Cys Ser Ile Gly Ser Thr
        195                 200                 205

Val Asp Gln Ala Asn Glu Ser Ile Val Ala Lys Thr Thr Val Thr Val
    210                 215                 220

Pro Ser Asp Gly Gly Pro Ile Glu Ala Val Ser Thr Ile Glu Thr Leu
225                 230                 235                 240

Pro Ser Trp Thr Arg Ser Arg Gly Lys Ser Gly Pro Leu Gln Pro Val
                245                 250                 255

Asn Ser Asp Ser Ala Leu Asn Ser Arg Pro Leu Glu Pro Arg Thr Asp
            260                 265                 270

Thr Asp Asn Leu Gly Thr Pro Gln Asn Thr Gly Gly Met Arg Leu His
        275                 280                 285

Asp Phe Val Ser Lys Thr Val Ile Lys Pro Glu Ser Cys Val Pro Cys
    290                 295                 300

Gly Lys Arg Ile Lys Phe Gly Lys Leu Ser Leu Lys Cys Arg Asp Cys
305                 310                 315                 320

Arg Leu Val Ser His Pro Glu Cys Arg Asp Arg Cys Pro Leu Pro Cys
                325                 330                 335

Ile Pro Pro Leu Val Gly Thr Pro Val Lys Ile Gly Glu Gly Met Leu
            340                 345                 350

Ala Asp Phe Val Ser Gln Ala Ser Pro Met Ile Pro Ala Ile Val Val
        355                 360                 365

Ser Cys Val Asn Glu Ile Glu Gln Arg Gly Leu Thr Glu Ala Gly Leu
    370                 375                 380

Tyr Arg Ile Ser Gly Cys Asp Arg Thr Val Lys Glu Leu Lys Glu Lys
385                 390                 395                 400

Phe Leu Lys Val Lys Thr Val Pro Leu Leu Ser Lys Val Asp Asp Ile
                405                 410                 415

His Val Ile Cys Ser Leu Leu Lys Asp Phe Leu Arg Asn Leu Lys Glu
            420                 425                 430

Pro Leu Leu Thr Phe Trp Leu Ser Lys Ala Phe Met Glu Ala Ala Glu
        435                 440                 445

Ile Thr Asp Glu Asp Asn Ser Thr Ala Ala Met Tyr Gln Ala Val Ser
    450                 455                 460

Glu Leu Pro Gln Ala Asn Arg Asp Thr Leu Ala Phe Leu Met Ile His
465                 470                 475                 480

Leu Gln Arg Val Ser Gln Ser Pro Asp Thr Lys Met Asp Ile Ala Asn
                485                 490                 495
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```
Leu Ala Lys Val Phe Gly Pro Thr Ile Val Ala His Thr Val Pro Asn
            500                 505                 510

Pro Asp Pro Val Thr Met Phe Gln Asp Ile Lys Arg Gln Leu Lys Val
        515                 520                 525

Val Glu Arg Leu Leu Ser Leu Pro Leu Glu Tyr Trp Asn Gln Phe Met
    530                 535                 540

Met Val Asp Gln Glu Asn Ile Asp Ser Gln Arg Gly Asn Gly Asn Ser
545                 550                 555                 560

Thr Pro Arg Thr Pro Asp Val Lys Val Ser Leu Leu Gly Pro Val Thr
                565                 570                 575

Thr Pro Glu Phe Gln Leu Val Lys Thr Pro Leu Ser Ser Ser Leu Ser
            580                 585                 590

Gln Arg Leu Tyr Asn Leu Ser Lys Ser Thr Pro Arg Phe Gly Asn Lys
        595                 600                 605

Ser Lys Ser Ala Thr Asn Leu Gly Gln Gln Gly Lys Phe Phe Pro Ala
    610                 615                 620

Pro Tyr Leu Lys
625
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<210> 5
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<212> DNA
<213> Caenorhabditis elegans

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<222> (1)..(2046)

<400> 5

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Met Lys Ser Ser Thr Ser Lys Glu Lys Val Cys Gly Glu Asn Ser Arg
  1               5                  10                  15

cac att ttc aac atg att cta aac tca cag cga ccg caa ttc gat att   96
His Ile Phe Asn Met Ile Leu Asn Ser Gln Arg Pro Gln Phe Asp Ile
             20                  25                  30

aag gat ata gga atg ttt cat ttg att gat gag att gag cgt ctc cgc   144
Lys Asp Ile Gly Met Phe His Leu Ile Asp Glu Ile Glu Arg Leu Arg
         35                  40                  45

aag ctg tgg aaa gat tcc gag gaa tcc aaa aag cgg ctg aat gca gat   192
Lys Leu Trp Lys Asp Ser Glu Glu Ser Lys Lys Arg Leu Asn Ala Asp
     50                  55                  60
```

```
atg aga gag gcc gaa gaa gca ctt gca aaa gct cgc aag aag ctg gca   240
Met Arg Glu Ala Glu Glu Ala Leu Ala Lys Ala Arg Lys Lys Leu Ala
 65                  70                  75                  80

atg ttc gat atc gat gtc aaa gac act cag aaa cat tta cgc gcg ttg   288
Met Phe Asp Ile Asp Val Lys Asp Thr Gln Lys His Leu Arg Ala Leu
                85                  90                  95

atg gaa gaa aat aag gcg ttg aag ctc gat cta aac gtc tac gag act   336
Met Glu Glu Asn Lys Ala Leu Lys Leu Asp Leu Asn Val Tyr Glu Thr
            100                 105                 110

cgt gaa aag cag ctg aaa gat gcg atg aag aac ggt ata ttc aat agt   384
Arg Glu Lys Gln Leu Lys Asp Ala Met Lys Asn Gly Ile Phe Asn Ser
        115                 120                 125

ctc acc aag gaa gac cgc gat cag ttc aag ttt ctt cac gag cca ctg   432
Leu Thr Lys Glu Asp Arg Asp Gln Phe Lys Phe Leu His Glu Pro Leu
    130                 135                 140

gtc cgg aca tac tcg aaa cgg gtg cag cag agg cat cca cat ttg atg   480
Val Arg Thr Tyr Ser Lys Arg Val Gln Gln Arg His Pro His Leu Met
145                 150                 155                 160

gag gac aca cag gac gat gag gac gat agt gag gtg gat tac gat gaa   528
Glu Asp Thr Gln Asp Asp Glu Asp Asp Ser Glu Val Asp Tyr Asp Glu
                165                 170                 175

act gga gac agt ttc gag gaa gtt att cat ttg cgc aat gga aga gag   576
Thr Gly Asp Ser Phe Glu Glu Val Ile His Leu Arg Asn Gly Arg Glu
            180                 185                 190

gtc aga aga agc tca gct gct gga aac gca gtt ggt ggc aag cgg aga   624
Val Arg Arg Ser Ser Ala Ala Gly Asn Ala Val Gly Gly Lys Arg Arg
        195                 200                 205

agc gcg tca gca cat gcg att act gct gct gcc aat tcg aag agg agc   672
Ser Ala Ser Ala His Ala Ile Thr Ala Ala Ala Asn Ser Lys Arg Ser
    210                 215                 220

aga agc cgt gtt atg aca gct act ata gat gaa gag ccg aat gag ggt   720
Arg Ser Arg Val Met Thr Ala Thr Ile Asp Glu Glu Pro Asn Glu Gly
225                 230                 235                 240

ggt aca cct cca aaa aga tgc cgt gat gat ggt tct aca cct cat caa   768
Gly Thr Pro Pro Lys Arg Cys Arg Asp Asp Gly Ser Thr Pro His Gln
                245                 250                 255

gaa atg aca act acc acc act acc acc acc acc act act att cat aac   816
Glu Met Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Ile His Asn
            260                 265                 270

tct cga gct cag aac cag gac ccg cca cgt gtc tca ctt cac cgc cag   864
Ser Arg Ala Gln Asn Gln Asp Pro Pro Arg Val Ser Leu His Arg Gln
        275                 280                 285
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ctc acc cgc agg agc ttg agc tgt gga agt att cca tca tgc gat caa   912
Leu Thr Arg Arg Ser Leu Ser Cys Gly Ser Ile Pro Ser Cys Asp Gln
    290                 295                 300

aca cca gga caa acc aca aat aac atc ggc ctc ggc atg tcg tcc gcc   960
Thr Pro Gly Gln Thr Thr Asn Asn Ile Gly Leu Gly Met Ser Ser Ala
305                 310                 315                 320

att ctc acc aaa agc aca ctt gat atc cga acc ctg aaa cgt ggc acg   1008
Ile Leu Thr Lys Ser Thr Leu Asp Ile Arg Thr Leu Lys Arg Gly Thr
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ccg gcg tgg act aat gga aca act cgt gac atc gca atg aga cca cac   1056
Pro Ala Trp Thr Asn Gly Thr Thr Arg Asp Ile Ala Met Arg Pro His
            340                 345                 350

acg ttt ata gag gca gga atc aaa gcg atg cga aaa tgc gac aaa tgt   1104
Thr Phe Ile Glu Ala Gly Ile Lys Ala Met Arg Lys Cys Asp Lys Cys
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Ala Thr Ala Leu Lys Leu Ala Thr Ser Met Lys Cys Arg Asp Cys His
    370                 375                 380

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Gln Val Val His Arg Ser Cys Cys Asn Lys Leu His Leu Pro Cys Ile
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cca cgc ccc aag acg atg atg acg ccg aaa tcc gca tta cgt gga gcc   1248
Pro Arg Pro Lys Thr Met Met Thr Pro Lys Ser Ala Leu Arg Gly Ala
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Lys Pro Gly Ala Gly Glu Phe Arg Leu Gln Asp Leu Cys Thr Ser Ala
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Lys Pro Met Ile Pro Ala Ala Val Ile His Cys Val Val Ala Leu Glu
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gct cgt gga ctc acg cag gaa ggt att tac cgc gtt cct ggg cag gtt   1392
Ala Arg Gly Leu Thr Gln Glu Gly Ile Tyr Arg Val Pro Gly Gln Val
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aga act gtc aat gtg ctt ttg gac gag ttg aga tca aaa acg gta ccc   1440
Arg Thr Val Asn Val Leu Leu Asp Glu Leu Arg Ser Lys Thr Val Pro
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aac gtg ggc ctt cac gac gtt gag gtc atc aca gac acc ttg aaa cgg   1488
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ttc cta aga gat ctt aaa gac ccg ttg atc cca aga acg tct cgt caa   1536
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cgt ctc gcc ctg aat cga gtg atc tgt gag ctc ccc caa gcg aat cga   1632
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Ser Ser Arg Asn Lys Met Asn Cys Glu Ala Met Ala Arg Met Val Ala
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Pro Ala Val Met Gly His Pro Val Lys Gln Ser Gln Ser Gln Ala Ile
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gct ggc aga gat gcc acg gat tgc cat cga gcg atg act gct cta ttt   1824
Ala Gly Arg Asp Ala Thr Asp Cys His Arg Ala Met Thr Ala Leu Phe
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gaa ttt gat gat gta tat tgg caa cga ttc cta ggg aca tct gca gtt   1872
Glu Phe Asp Asp Val Tyr Trp Gln Arg Phe Leu Gly Thr Ser Ala Val
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tcc atg gct tct aat caa att gaa acg gct cga cat cag gac aat ttt   1920
Ser Met Ala Ser Asn Gln Ile Glu Thr Ala Arg His Gln Asp Asn Phe
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Thr Pro Leu Leu Ala Arg Ser Ala Asn Ala Thr Arg Ala Arg Gly Ala
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His Ile Phe Asn Met Ile Leu Asn Ser Gln Arg Pro Gln Phe Asp Ile
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Lys Asp Ile Gly Met Phe His Leu Ile Asp Glu Ile Glu Arg Leu Arg
          35                  40                  45

Lys Leu Trp Lys Asp Ser Glu Glu Ser Lys Lys Arg Leu Asn Ala Asp
      50                  55                  60

Met Arg Glu Ala Glu Glu Ala Leu Ala Lys Ala Arg Lys Lys Leu Ala
 65                  70                  75                  80

Met Phe Asp Ile Asp Val Lys Asp Thr Gln Lys His Leu Arg Ala Leu
                 85                  90                  95

Met Glu Glu Asn Lys Ala Leu Lys Leu Asp Leu Asn Val Tyr Glu Thr
            100                 105                 110

Arg Glu Lys Gln Leu Lys Asp Ala Met Lys Asn Gly Ile Phe Asn Ser
        115                 120                 125

Leu Thr Lys Glu Asp Arg Asp Gln Phe Lys Phe Leu His Glu Pro Leu
    130                 135                 140

Val Arg Thr Tyr Ser Lys Arg Val Gln Gln Arg His Pro His Leu Met
145                 150                 155                 160

Glu Asp Thr Gln Asp Asp Glu Asp Asp Ser Glu Val Asp Tyr Asp Glu
                165                 170                 175

Thr Gly Asp Ser Phe Glu Glu Val Ile His Leu Arg Asn Gly Arg Glu
            180                 185                 190

Val Arg Arg Ser Ser Ala Ala Gly Asn Ala Val Gly Gly Lys Arg Arg
        195                 200                 205

Ser Ala Ser Ala His Ala Ile Thr Ala Ala Ala Asn Ser Lys Arg Ser
    210                 215                 220

Arg Ser Arg Val Met Thr Ala Thr Ile Asp Glu Glu Pro Asn Glu Gly
225                 230                 235                 240

Gly Thr Pro Pro Lys Arg Cys Arg Asp Asp Gly Ser Thr Pro His Gln
                245                 250                 255

Glu Met Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Ile His Asn
            260                 265                 270

Ser Arg Ala Gln Asn Gln Asp Pro Pro Arg Val Ser Leu His Arg Gln
        275                 280                 285

Leu Thr Arg Arg Ser Leu Ser Cys Gly Ser Ile Pro Ser Cys Asp Gln
    290                 295                 300

Thr Pro Gly Gln Thr Thr Asn Asn Ile Gly Leu Gly Met Ser Ser Ala
305                 310                 315                 320

Ile Leu Thr Lys Ser Thr Leu Asp Ile Arg Thr Leu Lys Arg Gly Thr
                325                 330                 335
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Pro Ala Trp Thr Asn Gly Thr Thr Arg Asp Ile Ala Met Arg Pro His
            340                 345                 350

Thr Phe Ile Glu Ala Gly Ile Lys Ala Met Arg Lys Cys Asp Lys Cys
        355                 360                 365

Ala Thr Ala Leu Lys Leu Ala Thr Ser Met Lys Cys Arg Asp Cys His
    370                 375                 380

Gln Val Val His Arg Ser Cys Cys Asn Lys Leu His Leu Pro Cys Ile
385                 390                 395                 400

Pro Arg Pro Lys Thr Met Met Thr Pro Lys Ser Ala Leu Arg Gly Ala
                405                 410                 415

Lys Pro Gly Ala Gly Glu Phe Arg Leu Gln Asp Leu Cys Thr Ser Ala
            420                 425                 430

Lys Pro Met Ile Pro Ala Ala Val Ile His Cys Val Val Ala Leu Glu
        435                 440                 445

Ala Arg Gly Leu Thr Gln Glu Gly Ile Tyr Arg Val Pro Gly Gln Val
    450                 455                 460

Arg Thr Val Asn Val Leu Leu Asp Glu Leu Arg Ser Lys Thr Val Pro
465                 470                 475                 480

Asn Val Gly Leu His Asp Val Glu Val Ile Thr Asp Thr Leu Lys Arg
                485                 490                 495

Phe Leu Arg Asp Leu Lys Asp Pro Leu Ile Pro Arg Thr Ser Arg Gln
            500                 505                 510

Glu Leu Ile Val Ala Ala Asn Leu Tyr Ser Thr Asp Pro Asp Asn Gly
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Arg Leu Ala Leu Asn Arg Val Ile Cys Glu Leu Pro Gln Ala Asn Arg
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Asp Thr Leu Ala Tyr Leu Phe Ile His Trp Arg Lys Val Ile Ala Gln
545                 550                 555                 560

Ser Ser Arg Asn Lys Met Asn Cys Glu Ala Met Ala Arg Met Val Ala
                565                 570                 575

Pro Ala Val Met Gly His Pro Val Lys Gln Ser Gln Ser Gln Ala Ile
            580                 585                 590

Ala Gly Arg Asp Ala Thr Asp Cys His Arg Ala Met Thr Ala Leu Phe
        595                 600                 605

Glu Phe Asp Asp Val Tyr Trp Gln Arg Phe Leu Gly Thr Ser Ala Val
    610                 615                 620

Ser Met Ala Ser Asn Gln Ile Glu Thr Ala Arg His Gln Asp Asn Phe
625                 630                 635                 640
```

```
Ala Leu Cys Asp Arg Ser Ile Leu Gly Pro Val Thr Thr Ser Pro Ala
                645                 650                 655

Thr Pro Leu Leu Ala Arg Ser Ala Asn Ala Thr Arg Ala Arg Gly Ala
            660                 665                 670

His Leu Leu Gly Ser Met Phe His Asp
        675                 680
```

Claims

1. Human poypeptide designated Cyk-4, which is a GTPase activating protein (GAP) for Rho family of GTPases, with the amino acid sequence as set forth in SEQ ID NO:2 or with the amino acid sequence encoded by a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:1.

2. Murine Cyk-4 poypeptide designated Cyk-4, which is a GTPase activating protein (GAP) for Rho family of GTPases, with the amino acid sequence as set forth in SEQ ID NO:4 or with the amino acid sequence encoded by a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:3.

3. An isolated DNA molecule comprising a polynucleotide with the nucleotide sequence as set forth in SEQ ID NO:1 encoding human Cyk-4 polypeptide, or an isolated DNA molecule encoding human Cyk-4 polypeptide comprising a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:1.

4. An isolated DNA molecule comprising a polynucleotide with the nucleotide sequence as set forth in SEQ ID NO:3 encoding murine Cyk-4 polypeptide, or an isolated DNA molecule encoding murine Cyk-4 polypeptide comprising a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:3.

5. An antibody which is specifically reactive with an epitope of the human Cyk-4 polypeptide of claim 1.

6. An antibody which is specifically reactive with an epitope of the murine Cyk-4 polypeptide of claim 2.

7. A method for identifying a compound capable of modulating cytokinesis, wherein the compound's ability to modulate the function of CYK- is determined.

8. The method of claim 7 wherein the compound's ability to promote GTP hydrolysis by a Rho family GTPase is determined by incubating a substrate selected from the members of the Rho family GTPases with GTP for a period of time sufficient to allow saturation of the substrate's GTP binding sites, adding Cyk-4 and allowing it to react in the presence or absence of the test compound, and determining the amount of hydrolized GTP.

9. The method of claim 7 wherein the compound's ability to inhibit Cyk-4 function is determined by determining the compound's ability to interfere with the biochemical interaction of CYK-4 and a member of the MKLP1 subfamily.

10. A compound identified in the method of any one of claims 7 to 9 for use in cancer therapy.

Abstract

Mammalian CYK-4 polypeptides, which are key molecules required for cytokinesis, and DNA molecules encoding them. Screening methods based on the function of CYK-4 to promote GTP hydrolysis by Rho family GTPases can be employed to identify compounds interfering with cell division. Such compounds are useful in tumour therapy.

Fig. 1

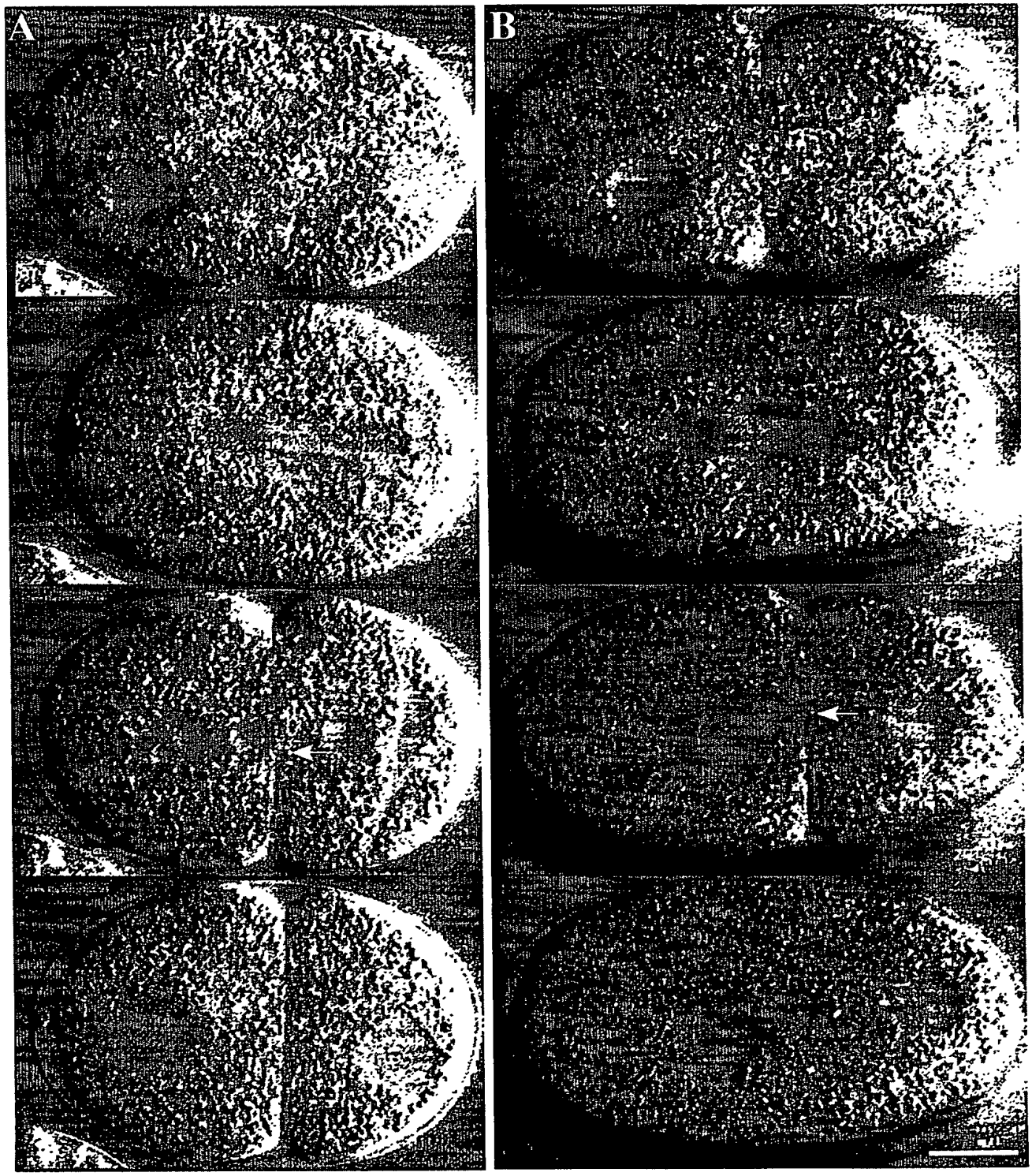


Fig. 2

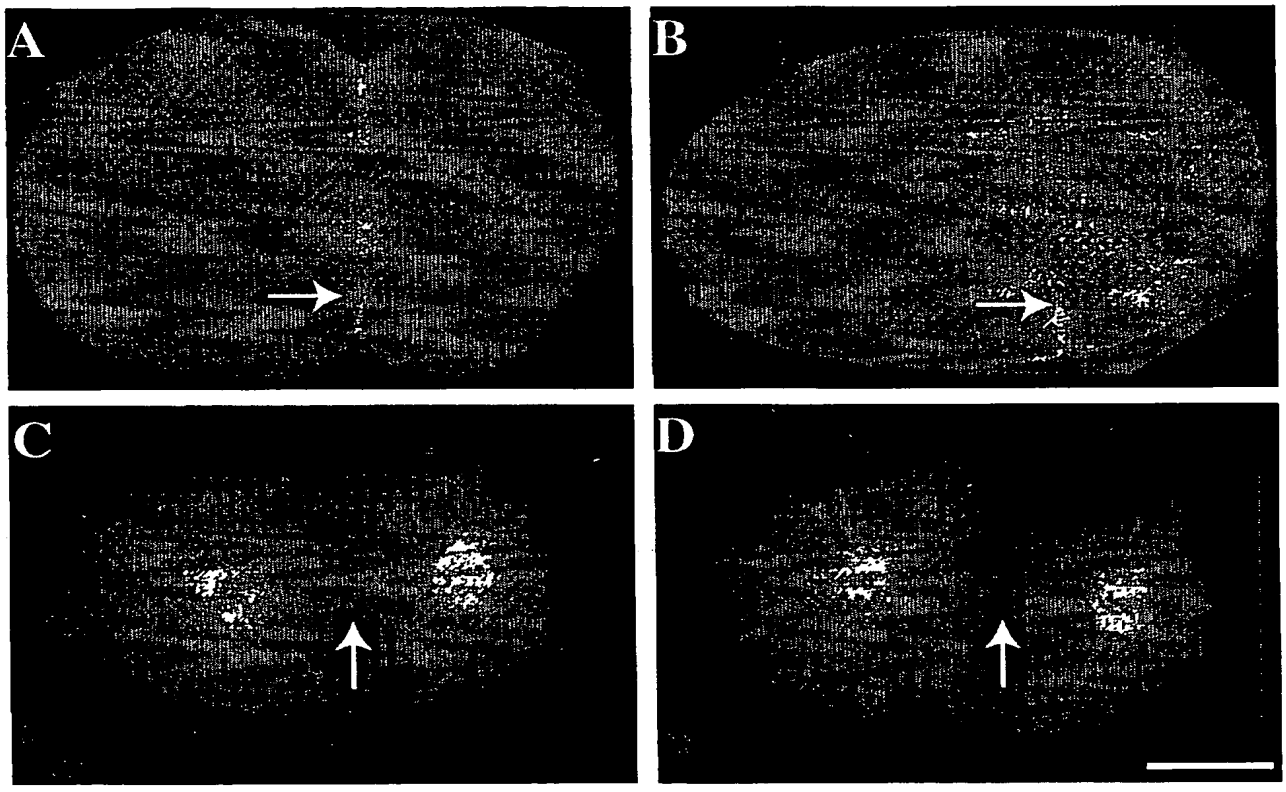
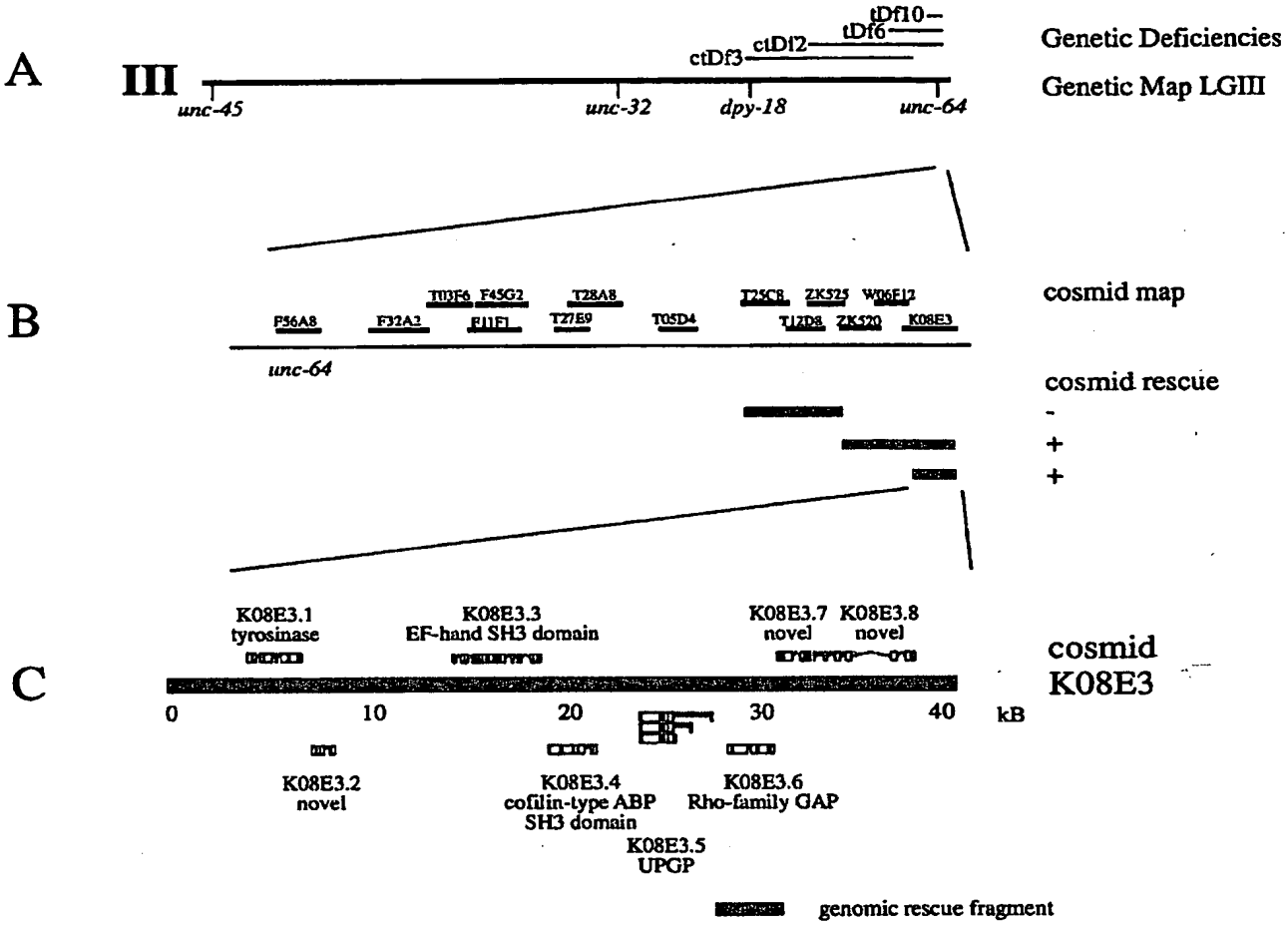


Fig. 3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W06E12
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Coiled-coil
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DAG/PE
binding
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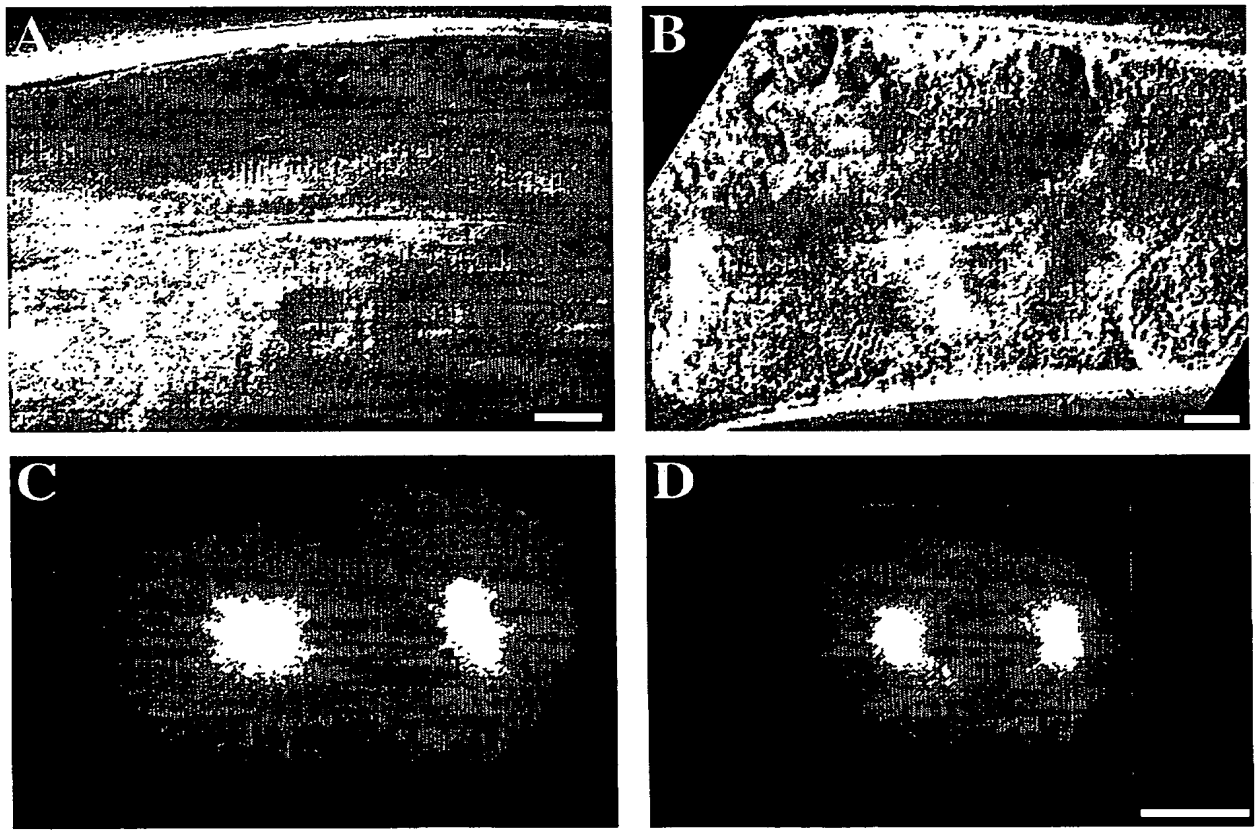
Fig. 4



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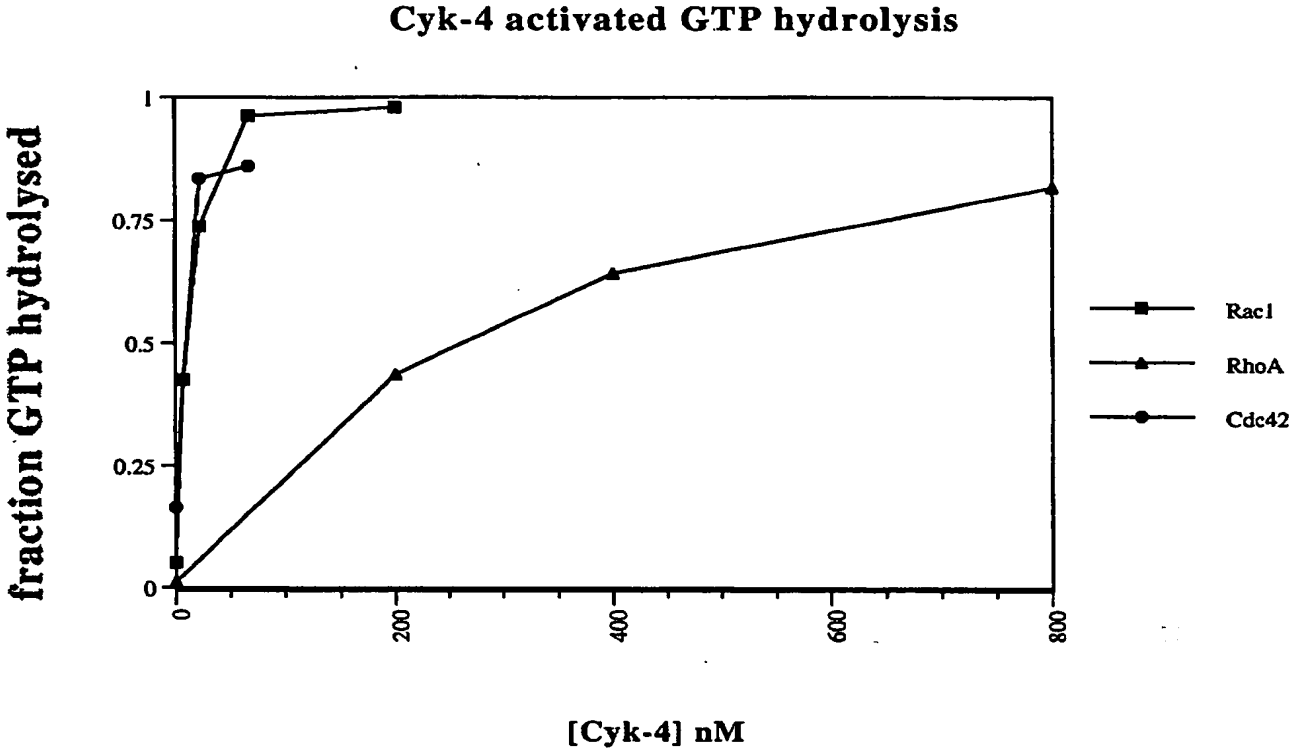
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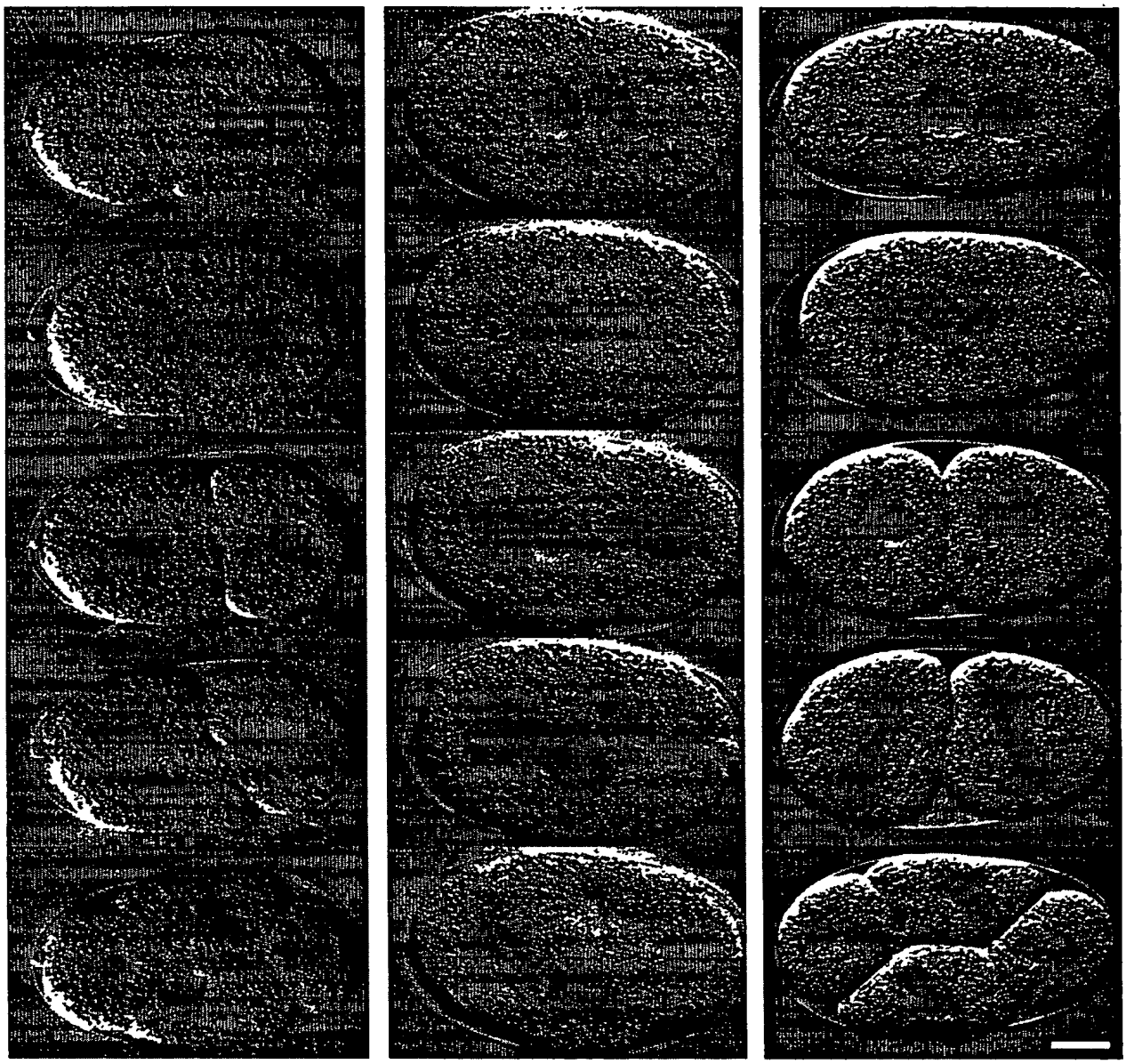
0.5
0.25
0
0
200
400
600
800
[Cyk-4] nM
Rac1
RhoA
Cdc42

Fig. 6



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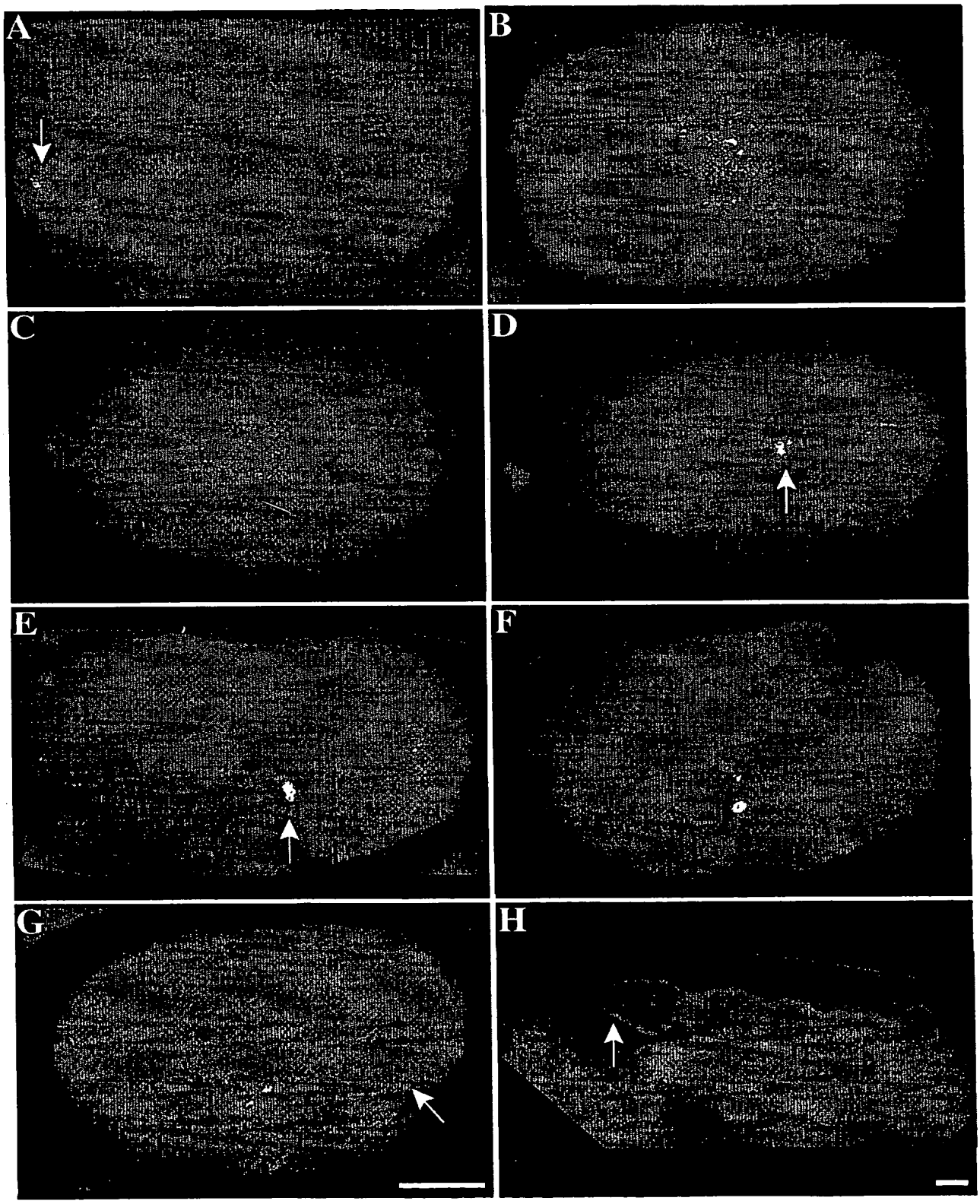


Fig. 8

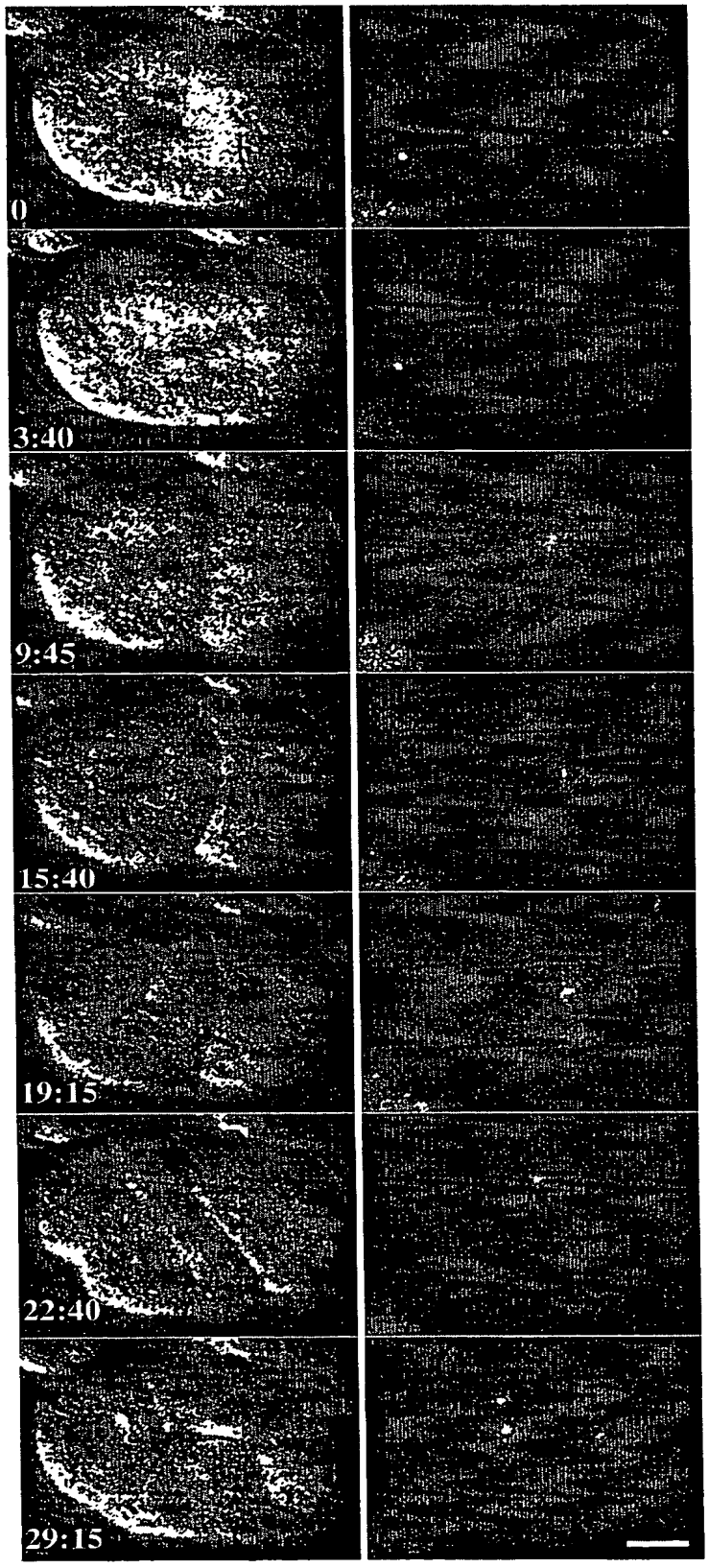


Fig. 9

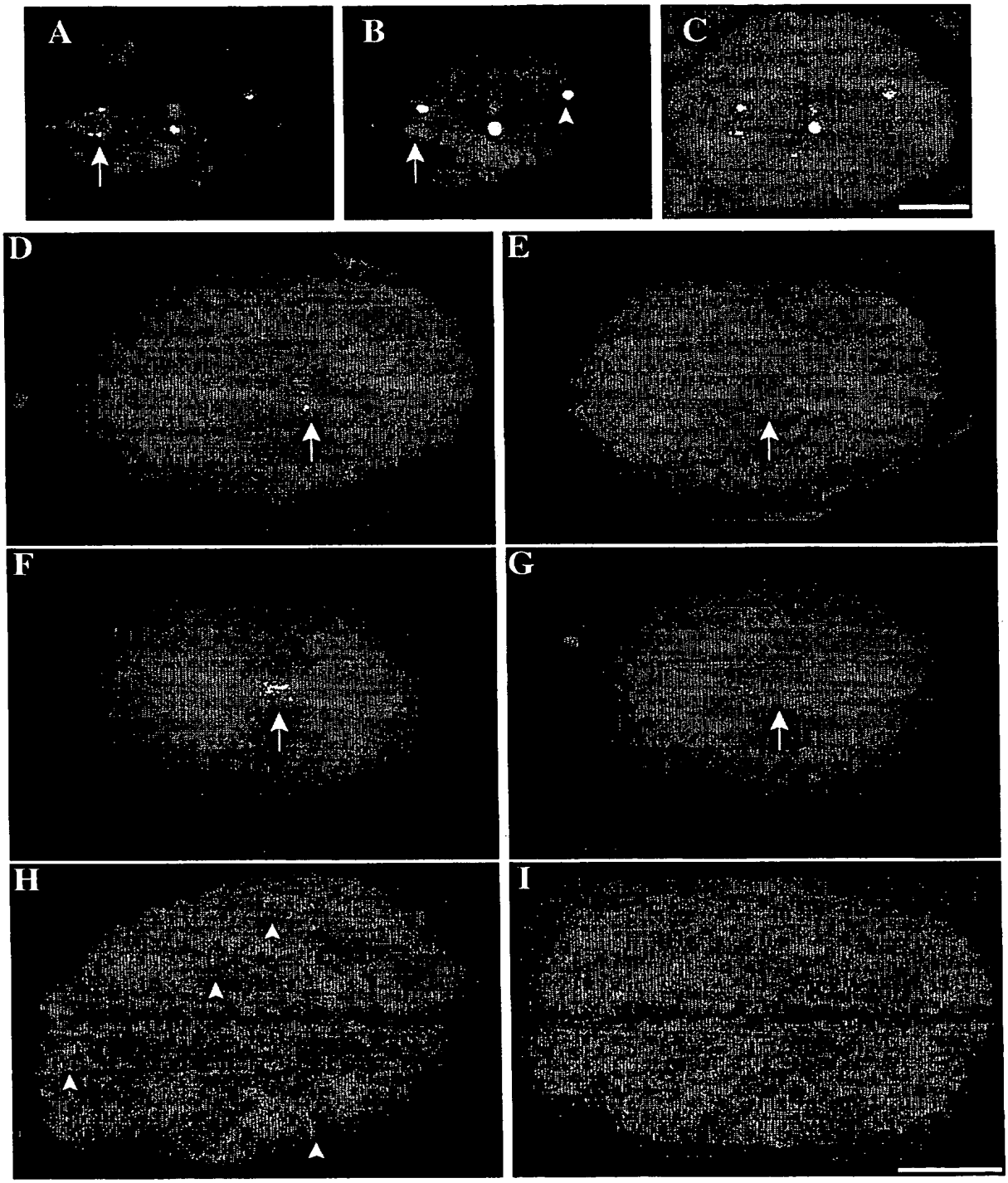
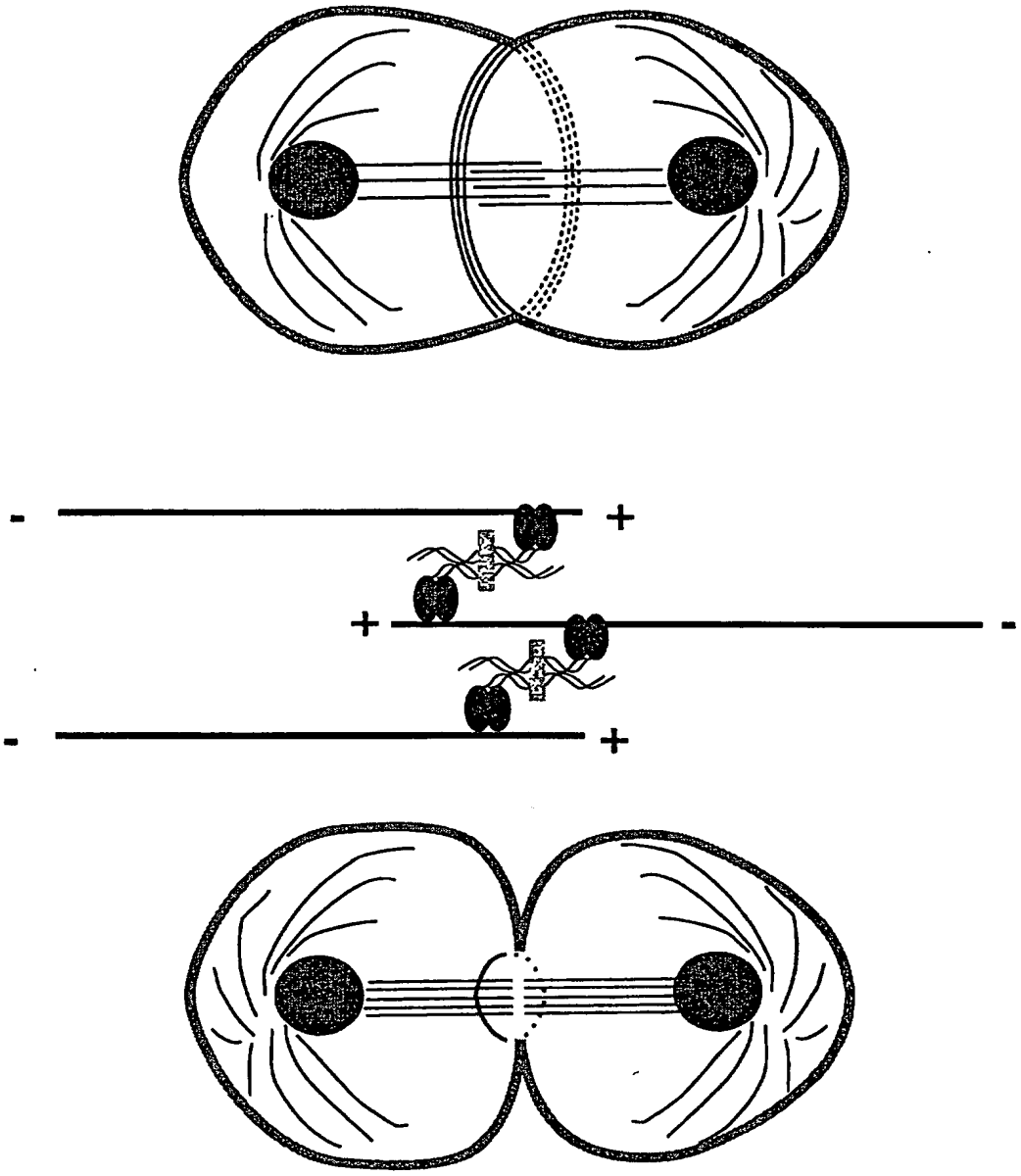


Fig. 10



Zen-4/CeMklp1 homodimer

Cyk-4